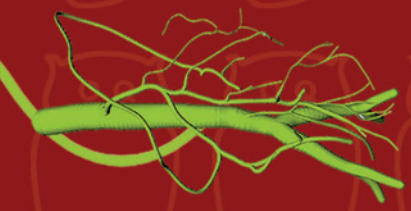
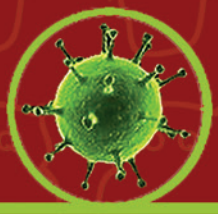
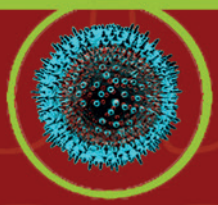
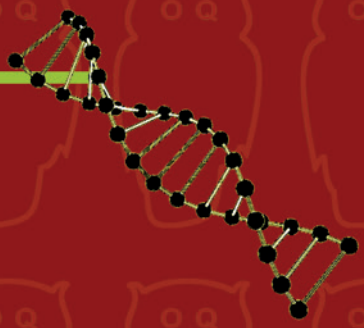
The Immune Response in Q Fever









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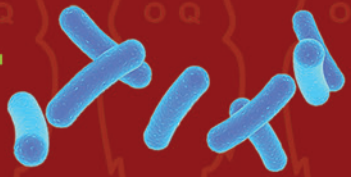

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Contents

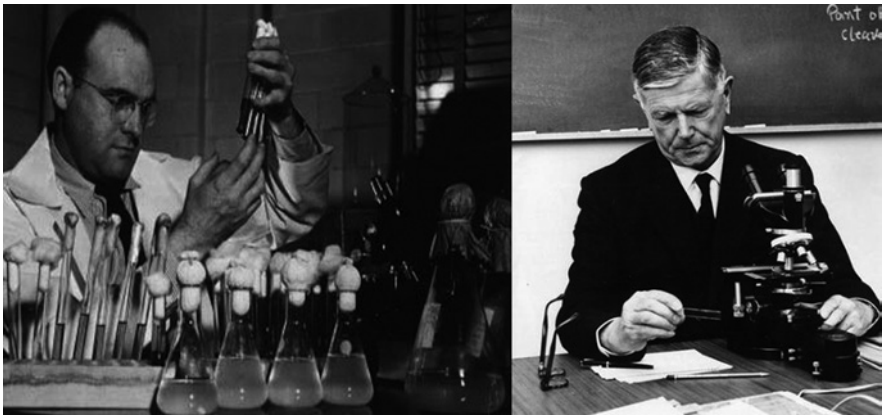
1

General introduction and outline of the thesis

A history of Q fever

What's in the name?

Q fever was first documented in 1937 in abattoir workers in Queensland, Australia [1]. The non-specific symptoms of fever, intense headache, malaise and anorexia led to search of infectious causes like typhus, typhoid and paratyphoid fever, and leptospirosis. When all the tests for these infections were negative, the hitherto unknown disease was designated Query (Q) fever [1]. This name has stuck to the disease ever since, even though the causative agent *Coxiella burnetii* was identified in the subsequent years by Cox and Burnet [2, 3]. The term 'Q fever' does not cover the differential clinical manifestations of the disease, but surely this name resonates well and the uncommon initial letter makes it stand out in lists of (infectious) diseases.



Harald Cox [Montana, USA] (Left) and Frank Marfarlane Burnet [Queensland, Australia] (Right)

Coxiella burnetii, I presume?

Along with the first documentation of Q fever, the causative agent of the disease was isolated from the blood and urine of symptomatic Q fever patients, by serial passage in guinea pigs [2]. It appeared to be typical Rickettsiae in smears from the spleens of infected mice [2]. This investigation of the bacterium in Australia coincided with the isolation of a previously unknown agent from ticks collected in Nine Mile (Montana, USA) during a field study on Rocky Mountain spotted fever. The initial American reports on the organism rejected it as a 'true' bacterium and assigned it viral characteristics, since it was easily filterable and unable to grow in cell-free culture [3]. After injection into guinea pigs, it produced a febrile illness that was different from Rocky Mountain spotted fever [3]. The link between the

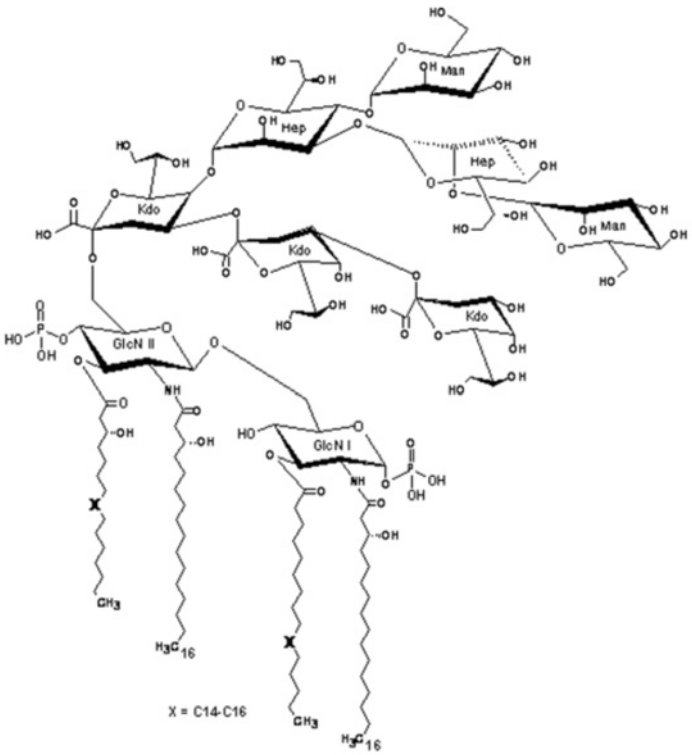
Australian and American researchers was made coincidently when an NIH Director visiting the Rocky Mountain Laboratory contracted infection with the Nine Mile agent [4]. In the report about this patient 'X', written by the NIH director himself, the author describes how he experienced signs and symptoms strikingly similar to Q fever. Subsequent cross protection studies confirmed that the Q fever and the Nine Mile agent were very likely the same pathogen [5]. It was proposed to name the organism *Coxiella burnetii* to credit both groups for their research. First being placed in the family Rickettsiaceae, *C. burnetii* has been reclassified after that, following advancing techniques of classifying organisms based on rRNA and genome sequencing. It was moved to the order Legionellales being genetically more related to members in this order, becoming a member of the family Coxiellaceae. The complete genome of the first isolate of *C. burnetii* in the USA, called the "Nine Mile strain", obtained from a tick, has been sequenced in 2003 [6].

Dr Livingstone, I presume?

The bacterium

Coxiella burnetii is a Gram-negative coccobacillus and its life-cycle contains two morphologically distinct forms [7]. The small-cell variant (SMV) is the extracellular form that resists environmental stress such as desiccation, heat and chemical exposure [8]. The SCV is metabolically inactive, with tightly packed chromatin. These characteristics make the bacterium able to survive for prolonged periods in the environment and spread easily, both in a natural outbreak [9], as in hypothetical circumstances when it would be used as a biological weapon [10]. In contrast, the large-cell variant (LCV) is metabolically and divisionally active, and develops after SCV has infected the host's immune cells, inside the monocytes or macrophages. The trigger for this differentiation seems to be the low pH inside the cytoplasmic

vacuole in which *C. burnetii* survives [11], which is described below in more detail. To complicate the matter, *C. burnetii* displays antigenic phase variation, having a phase I and phase II form, based on the structure of the lipopolysaccharide (LPS) molecules on the outer membrane of the bacterium. *C. burnetii* isolated from natural sources such as patients and animals have full-length LPS molecules with O antigen, and are referred to as phase I bacteria. Phase shift can occur in the laboratory when serial *in-vitro* passages of phase I bacteria in embryonated eggs or tissue culture results in a truncated LPS molecule on the membrane of *C. burnetii*. This form is referred to as the phase II bacterium. The phase II LPS molecule has the same lipid A as phase I, but lacks the *C. burnetii*-specific O antigen sugars virenose and dihydrohydroxystreptose [12]. In the Nine Mile strain, this aberrant phase II LPS is caused by large chromosomal deletion of genes involved in O-antigen sugar biosynthesis [13].



Chemical structure of LPS phase II from *Coxiella burnetii* in avirulent phase II.
Abbrevations: Man, D-mannose; Hep, D-glycero- D-manno-heptose; Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid. GlcN, D-glucosamine. (Adapted from Toman et al. Coxiella burnetii *glycomics and proteomics--tools for linking structure to function. Ann N Y Acad Sci. 2009;1166:67-78)*

This phase shift is associated with loss of virulence: phase I bacteria are virulent in humans and in animal models, while phase II bacteria do not cause disease in immune-competent animals [14]. Moreover, vaccines that are made from inactivated phase II bacteria do not protect against subsequent infection; so phase I bacteria are used in vaccines [15-17].

The impact of this phase variation in *C. burnetii* on serological response of the infected host needs to be mentioned here. Serology of Q fever concerns with antibodies against both phase I and phase II bacteria. Counter-intuitively, during an acute infection, antibodies against phase II are first detected, later followed by antibodies against phase I antigens. In chronic infection, antibody titres to phase I antigens are persistently present, which has important consequences for the diagnosis of chronic Q fever, as discussed later.

Q fever as a zoonosis

C. burnetii is prevalent worldwide, with the exception of New-Zealand [18, 19], and human Q fever outbreaks are reported in many countries [20-25]. The recent outbreak in the Netherlands from 2007 to 2010 was the largest ever described, with more than 4,000 human cases notified, and estimates of 40,000 infected individuals, based on *C. burnetii* seroprevalence numbers among screened patients at high-risk for chronic Q fever [26].

Q fever is a zoonosis, with natural reservoirs in a wide range of wild and domestic animals. The most common reservoirs leading to human outbreaks, however, are domestic ruminants, mostly goats and sheep [20, 27, 28]. These animals do not suffer from overt disease, but *C. burnetii* can replicate massively in placentas of pregnant animals, often resulting in abortion, premature birth or stillbirth. During parturition, high amounts of bacteria spread into the environment, potentially leading to human infections. Inhalation of aerosols containing *C. burnetii*, derived from birth products, are the main route of transmission [19]. Moreover, *C. burnetii* is shed in milk, urine and faeces of infected animals and human infections from ingestion of contaminated dairy products has been described [29]. Traditionally, farmers, veterinarians and abattoir workers are most at risk for Q fever. The Dutch outbreak in goat farms occurred in densely-populated urban areas, affecting people living or recreating in the vicinity of the infected farms.

Q fever, the disease

Acute Q fever

Initial infection with *C. burnetii* can lead to symptoms of acute Q fever, but is asymptomatic in >50% of people, in which case it is detected retrospectively by the presence of anti-*C. burnetii* antibodies [20, 30]. Acute Q fever usually presents as a

self-limiting flu-like illness, after 7-21 days of incubation, and can be accompanied by other symptoms such as headache, fatigue, myalgia and respiratory symptoms. *C. burnetii* DNA can be detected in serum in the early acute phase of the disease, using poly- merase chain reaction (PCR). However, serology plays a central role in diagnosing acute Q fever. As described before, *C. burnetii* exhibits antigenic variation with phase I and phase II LPS, and antibodies against both phases are detected separately in serological assays. Antibodies are detectable within 1-2 weeks after start of symptoms of acute Q fever. The appearance of anti-phase II IgM (and IgG) antibodies accompanied by lower levels of anti-phase I antibodies are considered diagnostic of acute Q fever. Immunofluorescense (IFA) is the reference method, but other techniques are also used, i.e. complement fixation (CFT) and enzyme-linked immunosorbent assay (ELISA).

The diagnosis is often missed because symptoms are non-specific and can resolve without treatment. Antibiotics, preferably doxycycline 200 mg/day for 2 weeks, can be administered to shorten duration of symptoms [31]. A more serious presentations of acute Q fever is that of an atypical pneumonia, for which hospital admission can be necessary, and is estimated to occur in 2% of cases [30]. It should be noted that the necessity for hospital admission was much higher in the beginning of the epidemic in the Netherlands [32]. An overall mortality rate of acute Q fever of 1% is reported in France [19, 33]. Other rare manifestations of acute Q fever, including severe acute hepatitis, pericarditis, myocarditis and menin-go-encephalitis have been described [19]. One of the important long-lasting sequelae of acute Q fever is (post-) Q fever fatigue syndrome (QFS) [34, 35], which is reported in over 20% of patients with acute Q fever [36, 37]. These patients suffer from incapacitating fatigue that lasts at least 6 months after the acute episode, which can be accompanied by a complex of other complaints among which are nausea, headache, dizziness, blurred vision, joint and muscle pain, (night)sweats and dyspnoea during exercise [38]. Conventional laboratory or microbiological parameters, however, do not discriminate QFS from an uncomplicated past Q fever episode. The clinical presentation of QFS resembles chronic fatigue syndrome (CFS) and fatigue after infection with pathogens like *Borrelia burgdorferi*. The aetiology of QFS is not elucidated yet, although it has been suggested that non-viable *Coxiella* antigens persisting in bone marrow after infection result in chronic immune stimulation [39, 40], definite proof of this is lacking. Moreover, cytokine dysregulation in individuals with predisposing genetic polymorphisms in immune-related genes has been linked to QFS [41, 42].

Chronic Q fever

Regardless of the initial presentation, *C. burnetii* can establish a persistent infection that may present after months to years and cause chronic disease (chronic Q fever).

Common manifestations of chronic Q fever are endocarditis, mycotic vascular aneurysm, and vascular prosthesis infection; all of these are life-threatening conditions. The diagnosis of chronic Q fever is often difficult. Symptoms are non-specific and can include low-grade fever, weight loss, night sweats, hepato-splenomegaly and a persistently raised erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). Laboratory diagnosis of persistent infection relies on serology, polymerase chain reaction (PCR) and culture. While PCR and culture on valvular or vascular tissue are highly sensitive, tissue is only obtained when conventional surgery is carried out. On blood specimens, PCR and culture have a low sensitivity for chronic Q fever. Serology is therefore essential for diagnosis. Persisting high IgG antibody titres against *C. burnetii* phase I, and to a lesser extent phase II, are indicative of chronic *C. burnetii* infection. Cut-off anti-phase I IgG titre of >1:800 or 1:1600 based on a French in-house IFA, and >1:1024 based on a commercially available IFA (Focus Diagnostics), are considered for the diagnosis of chronic Q fever [43-45]. High phase I titres along with presence of definite endocarditis according to the modified Duke criteria [46] are considered confirmative of chronic Q fever endocarditis [47, 48]. Infected aneurysms or vascular prostheses can be identified by imaging techniques, most notably computed tomography (CT) and ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET/CT) [49, 50].

Treatment of chronic Q fever is long and cumbersome. Antibiotics should be administered for at least 18 months or, in case of a valvular/vascular prosthesis, for at least 24 months [51]. Surgical intervention to replace an infected vascular aneurysm/graft or cardiac valve is often necessary, either in the acute situation of symptomatic aortic aneurysm or heart failure, or when a patient does not improve on antibiotics [52-54].

Host factors predisposing to chronic Q fever

Individuals at risk for developing chronic Q fever after infection with *C. burnetii* are patients with pre-existing cardiac valvulopathy (including prosthetic valve) and vascular aneurysms or grafts. Other reported risk factors are pregnancy, older age and renal insufficiency [52, 54-58]. In the large Dutch Q fever outbreak, it became apparent that only a minority (7.8%) of patients with cardiac valvular risk-factors developed chronic Q fever after (serological evidence of) infection with *C. burnetii* [59]. For patients with pre-existing vascular aneurysms or prosthesis, this proportion was substantially higher (30.8%) [60]. It can be speculated that immunological host factors play an important role to prevent development of chronic Q fever in most individuals, but predispose a minority to clinical manifestations of chronic Q fever. Inefficient early recognition of the bacterium by the innate immune system followed by incomplete eradication and/or inadequate

initiation of adaptive immune responses may be pivotal factors in the development of chronic Q fever.

Indeed, immunosuppression is stated in the literature as a risk factor for chronic Q fever. Case studies describe chronic Q fever in patients with (haematologic) malignancies, or with liver- and bone marrow transplantation [58, 61-63]. A retrospective study of 102 Q fever endocarditis patients, reveals cancer as a risk factor [55], which may be related to the use of immuno-modulating cytotoxic treatment. A association between Q fever and the use of immunosuppressive drugs, including corticosteroids and methotrexate, is mentioned in three case reports [64-66], one of which describes a patient with chronic Q fever endocarditis [66]. It has also been suggested, based on animal and *in-vitro* experiments, that the persistence of *C. burnetii* in monocytes/macrophages is related to increased IL-10 [67-71]. This cytokine is able to down-modulate the microbicidal activity of macrophages. Capo et al. showed that peripheral blood mononuclear cells (PBMCs) from chronic Q fever patients spontaneously produced elevated levels of IL-10 [67]. In addition, Honstettre et al monitored cytokine production by PBMCs from acute Q fever patients and found that the amount of IL-10 correlated to the risk of development of chronic Q fever [68]. IL-10 was found to induce *C. burnetii* replication in naive human monocytes, probably mediated by decreased TNF production [69]. Neutralizing IL-10 in *in-vitro* cultured monocytes of chronic Q fever patients, restored the microbicidal activity of these cells [70]. It has been shown that transgenic mice that over-express IL-10 in the macrophage compartment fail to kill the bacteria and develop a persistent *C. burnetii* infection with similar pathology to human chronic Q fever patients [71].

Immune response in Q fever

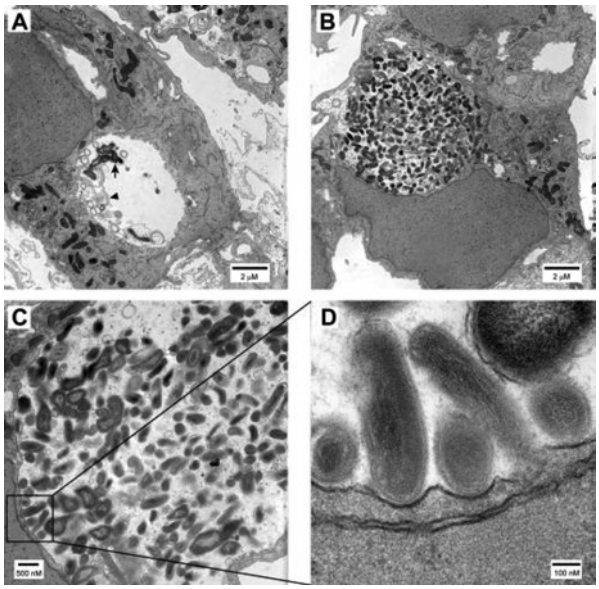
C. burnetii is an obligate intra-cellular bacterium that replicates in immune cells of the host, specifically cells of the mononuclear phagocyte lineage: monocytes and macrophages. After aerosol inhalation of the SCV form of *C. burnetii*, the bacterium targets the alveolar macrophages [72]. In an acute Q fever model in guinea pigs, immunohistochemistry confirmed the presence of *C. burnetii* organisms in infected animals in the lungs, liver, and spleen. Bacteria were most prominent in monocytes and macrophages but were also noted in other cell types, mainly pneumocytes in the lungs [73]. In human acute Q infection, dougnut granulomas consisting of macrophages and lymphocytes have been found in liver and bone marrow biopsy specimens [19].

It is likely that *C. burnetii* is trafficked by infected monocytes/macrophages to other tissue than the lungs. Interestingly, cardiac valves, aneurysmatic vascular wall or prosthesis and placenta are predilection sites for outgrowth and persistence of *C. burnetii*. In chronic Q fever, no granulomas are found in *C. burnetii*-infected

tissue such as heart valves, liver and aneurysms [19, 74]. In valvular tissues, *C. burnetii* is detected intracellularly, clustered as a single intracytoplasmic mass, almost exclusively in local macrophages [74]. Vegetations are difficult to detect using echocardiography in Q fever endocarditis [30, 75]. Somewhat contradictory, a recent histological study on *C. burnetii*-infected vascular wall tissue showed a necrotizing granulomatous response in four of seven patients with vascular chronic Q fever [76].

It has been demonstrated that *C. burnetii* phase I enters monocytes/macrophages using $\alpha_v\beta3$-integrin (CD51/CD61) as binding receptor [77, 78]. Binding to Toll-like receptor (TLR)4 has been proposed to be involved in actin rearrangement and phagocytosis [79]. TLR4 knockout mice showed only transient granuloma formation after inoculation with *C. burnetii*, in contrast to wild-type mice. However, TLR4 knockout mice did not show increased *C. burnetii* survival inside macrophages *in vitro*, or a defect in bacterial clearance *in vivo* [79].

Interestingly, Zamboni et al. have shown that phase I LPS of *C. burnetii* is a poor immunogenic stimulus and functions as an antagonist of TLR4-dependent



Transmission electron micrographs of phase II *Coxiella*-infected Vero cells.
Cells were infected for 2 days (A) or 6 days (B–D), then examined via transmission electron microscopy. Panel A shows a spacious PV at 2 days post infection (dpi) harbouring a few Coxiella *organisms (arrow). Multilamellar bodies (arrowhead) are also present that may result from PV fusion with autophagosomes. By 6 dpi (B and C), the PV has enlarged to occupy a large portion of the host cell and contains numerous* Coxiella *that have entered the stationary phase of their growth cycle (Coleman et al., 2004). (Adapted from Voth&Heinzen, Lounging in a lysosome: the intracellular lifestyle of Coxiella burnetii. Cell Microbiol 2007;9(4):829-40)*

signalling in macrophages [80]. This results from the fact that the lipid A portion of *C. burnetii* LPS is a tetra-acylated structure, in contrast to e.g. *Escherichia coli* LPS which has a hexa-acylated lipid A portion which is a thousand times more potent as an endotoxin [81-83]. It has been suggested that the full-length LPS molecule of phase I *C. burnetii* serves as a shielding molecule, which masks antigens on its surface from immune recognition by other pattern recognition receptors (PRRs) of the host's immune cells [84], i.e., TLR2. Indeed, *C. burnetii* phase II - with short LPS molecules - stimulate macrophages through TLR2 [80]. Macrophages from TLR2 knockout mice were highly permissive to *C. burnetii* phase II growth, compared to either TLR4 knockout or wild-type mice [80].

Once inside cells, *C. burnetii* resides in an cytoplasmic vacuole – the parasitophorous vacuole (PV) – which closely resembles a phagolysosome, containing lysosomal enzymes and having a low pH (<5.0). During maturation of the PV, the vacuole increases in size till it almost completely fills the host cell cytoplasm [85]. During this process, the SCV converts to the LCV and becomes metabolically active and replicates. It is quite unique that *C. burnetii* can withstand the active acid hydrolases and degradative activity inside the PV [86]. It even replicates to high numbers and thrives in this hostile environment in which other bacteria are degraded [87]. *C. burnetii* promotes vacuole biogenesis and maintenance to survive, and is able to inhibit apoptosis of the host cell [88]. Authophagy, normally a mechanism of the innate immune system to remove intracellular pathogens, is induced by *C. burnetii* favouring the generation and maturation of the PV and thereby actually promoting intracellular replication [89, 90].

It is known that antibodies can provide protection against intracellular pathogens via a number of different mechanisms, including direct bactericidal activity, complement activation, opsonization, cellular activation via Fc or complement receptors, and antibody-dependent cellular cytotoxicity [91, 92].

However, the role of humoral responses in Q fever are not clear. Early studies on *C. burnetii* have shown that passive transfer of antibody protects guinea pigs against subsequent challenge with *C. burnetii* [93]. Other studies in mice confirmed that passive immunization of naïve mice with serum from vaccinated mice fully protected against challenge [17]. However, immune sera or B-cells of immunized mice that were transferred to SCID mice, conferred no protection against *C. burnetii* challenge, while T-cells did [17], emphasizing the importance of T cell-mediated immunity.

The high antibody titres that are found in chronic Q fever to phase I and phase II antigens are apparently not effective for *C. burnetii* clearance. It has even been made plausible that these high antibody titres are potential detrimental during chronic Q fever, because immune complexes can cause pathology such as

glomerulonephritis or vascular leukocytoclastic lesions [62]. In addition, conflicting results have been obtained on the effect of antibody opsonisation on the intracellular fate of *C. burnetii*. Desnues et al demonstrated that *C. burnetii* that was opsonized with IgG antibodies purified from chronic Q fever patients, favoured *C. burnetii* replication within human macrophages [94]. Shannon et al., however, found increased uptake of antibody-opsonized *C. burnetii* by macrophages, but did not observe subsequently impact on growth rate intracellularly [92, 95]. They found, however, that antibody- opsonization of *C. burnetii* led to increased maturation of dendritic cells and increased cytokine production. Surprisingly, both the Fc receptor, as well as complement receptors were redundant for passive immunization *in vivo*, as shown by equal protection of wildtype mice compared to FcR knockout as well as several different strains of complement-deficient mice [95].

Cell-mediated immune responses are important for the defence against intracellular bacteria, as shown for *Mycobacterium tuberculosis, Mycobacterium leprae, Listeria monocytogenes,* and *Legionella pneumophila* [92]. These intracellular bacteria infect professional antigen-presenting cells, resulting in antigen presentation on a MHC II molecule on the surface of the cells. In combination with T cell co-stimulatory molecules on the surface and the production of cytokines, this leads to activation of antigen-specific T cells, which are recruited to the site of infection. The latter produce pro-inflammatory cytokines such as interferon-gamma (IFN-γ) and tumour-necrosis factor (TNF), in turn leading to activation of macrophages to perform anti-microbial effector mechanisms that are able to kill the intracellular bacteria. In this process, granulomas are formed in infected tissue.

For *C. burnetii*, it has been shown in a series of experiments that when the cell-mediated immunity was suppressed in guinea pigs and mice by whole body irradiation, cortisone injection or pregnancy, the infection reactivated [96-98]. Andoh et al showed in a mouse model of acute Q fever that T cell-deficient mice and IFN-γ knockout mice were very susceptible to *C. burnetii* infection [99]. IFN-γ knockout succumbed to infection in an early stage, while T-cell deficient mice were not able to clear the infection within 4 weeks. Read et al reconstituted immuno-deficient SCID mice with CD4+ or CD8+ T cells, and depleted immunocompetent Balb/c mice of T cell subsets, thereby showing that CD4+ cells or CD8+ cells alone were able to control a primary infection with *C. burnetii* [100]. This suggests that both CD4+ and CD8+ T cells play an important role in host defense against *C. burnetii*, at least in the mouse.

In-vitro studies have shown that IFN-γ induces killing of *C. burnetii* in THP-1 monocytic cells, and inhibits growth of *C. burnetii* in mouse fibroblasts [101, 102]. Induction of reactive oxygen and nitrogen species (ROS and RNS, respectively) have been shown to be involved in the control of *C. burnetii* replication by IFN-γ

stimulation of primary mouse macrophages [103]. In humans, lymphocytes of vaccinees and people with a past infection proliferate in response to *C. burnetii* antigens *in vitro*, indicating long-lasting cell-mediated immune responses [104, 105]. IFN-γ production by T cells upon in-vitro *C. burnetii* stimulation was observed in immunized individuals [106].

Thus, cell-mediated immune responses contribute to clearance of *C. burnetii* infection and IFN-γ plays a key role in the control of *C. burnetii* replication.

Q fever vaccination

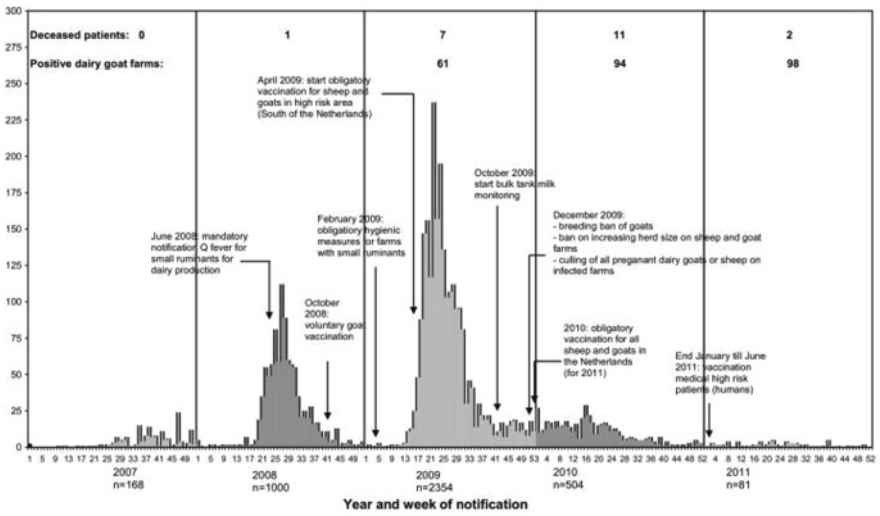
Development of a safe and effective Q fever vaccine started almost from the moment that the micro-organism was identified. These vaccine projects were not only initiated to protect people with occupational exposure to *C. burnetii* – the main focus was on workers in slaughterhouses in Australia – but were also the result from governmental concerns of *C. burnetii* as a biothreat [10]. Several attempts were unsuccessful sofar, including a live-attenuated phase II vaccine in Russia (107), a chloroform-methanol residue (CMR) extract vaccine in the USA [108, 109], and a trichloroacetic (TCA) extract in Slovakia [110]. These vaccines were either unsafe or found to be reactogenic [108, 110-112]. In addition, development of sub-unit protein vaccine had no success. A number of potential protective protein subunits were identified, but recombinant protein antigens expressed in *E. coli* showed limited efficacy [113-115].

The only commercially available vaccine has been licensed for use in Australia since 1989. This is a formaldehyde-inactivated whole-cell phase I vaccine (Q-vax; CSL Biotherapies, Australia), produced from the Henzerling strain of *C. burnetii* [116-118]. Studies have shown 97-100% protection in slaughterhouse workers for at least five years post-vaccination [116, 119, 120]. However, Q-vax induces side-effects in a significant proportion of vaccinees, similarly to the CMR and TCA extract vaccines. The most reported side-effects are swelling, erythema and tenderness at the site of vaccination, but also systemic mild symptoms such as headache and flu-like symptoms [118]. Individuals who had previous contact with *C. burnetii* need to be excluded from vaccination with Q-vax, because it has been reported that vaccination of people with pre-existing immunity against *C. burnetii* may lead to serious adverse reactions such as sterile abcesses and systemic hypersensitivity reactions [38, 121]. To identify pre-existing immunity, serology and skin testing is performed in all potential vaccinees before vaccination with Q-vax, according to the manufacturer's instruction [116]. A positive result in either one or both tests precludes vaccination.

A recent report on passive surveillance of the Australian national Q-fever vaccination campaign 2001-2004 in slaughterhouses, showed a very low number of reported adverse events after immunization (86/48,986;0.002%). These included

mostly local reactions, with only one serious adverse event [122]. There are only few reports of severe local reactions after Q-vax vaccination [123, 124]. It should be taken in account that all the data comes from vaccination of a predominantly young and healthy group of male slaughterhouse workers.

The Q fever outbreak in the Netherlands, which was related to dairy goat farms, spread among the human population living in close proximity to the farms with infected goats [125, 126]. The most important measures to prevent morbidity and mortality of human Q fever were taken in the veterinary sector, aiming at reducing the spread of *C. burnetii*. In the Netherlands, these included obligatory hygienic measures for farms with small ruminants, mandatory notification of *C. burnetii*-infected dairy goat and sheep farms, vaccination of all dairy goats and sheep on farms with more than 50 animals, bulk tank milk monitoring for *C. burnetii* DNA by PCR and culling of pregnant goats on infected farms [21]. Human vaccination with the Australian Q-vax vaccine, however, was decided to be of additional value in individuals particularly at high-risk for serious complications of Q fever, namely those with risk factors for development of chronic Q fever living in the Q fever high-incidence area [127]. This target population included patients with underlying cardiac valvulopathy (including prosthesis), a history of endocarditis, known (aortic) aneurysm or vascular prosthesis [127, 128]. The size of these target groups



Q fever notifications in the Netherlands 2007-2011 and measures taken.
(Adapted from Isken et al. Implementation of a Q fever vaccination program for high-risk patients in the Netherlands. Vaccine 31(23):2617-22)

was unknown and no attempts were made to estimate the number to be expected. Pre-vaccination screening – with *C. burnetii* serology and skin test – and vaccination took place at one municipal health centre in the high-incidence area between January and April 2011, with some catch-up vaccinations in June. The much larger than expected number of referred people overloaded the system and made implementation of the campaign difficult, forcing an up-scaling of staff. In the end, a total of 1786 patients were screened, of which 1370 were vaccinated.

Outline and aims of the thesis

In this thesis, we study the immune response to *Coxiella burnetii* in the light of chronic Q fever and Q fever vaccination, in order to define important aspects of the host's immunological defence against *C. burnetii*. The major goal of our research is to improve prevention, diagnosis and treatment of (chronic) Q fever. In addition, elucidating immunological aspects of the pathogenesis of Q fever disease opens up the possibilities of adjunctive immunosupportive treatment in selected patients. Knowledge on the applicability and immunogenicity of vaccination against *C. burnetii* in people with risk factors for chronic Q fever will help in future decisions on *C. burnetii* vaccination in outbreak situations.

- In **Part I** we look more closely into the recognition and response to *C. burnetii* by the immune system in humans and the association between disease outcome and genetic polymorphisms in immune-related genes. We investigated the specific interferon-gamma (IFN-γ) response pathway is investigated in chronic Q fever patients.
- In **Part II** we asked the question whether measurement of cell-mediated immune responses can be used as a diagnostic tool for Q fever.
- In **Part III** we investigated whether the use of frequently described drugs that suppresses the cell-mediated immune response in rheumatoid arthritis (RA) patients increases the risk of developing (acute or chronic) Q fever.
- In **Part IV**, we addressed the question to what extent Q fever vaccination is safe and immunogenic; in addition we investigated the coverage of the Dutch Q fever vaccination campaign.

PART I. Studies on the immune response against *C. burnetii* in (chronic) Q fever

The innate immune system provides the first line of host defense against microorganisms. After adequate recognition of the invading pathogen, host-defense mechanisms are activated, such as cytokine signaling and phagocytosis, which are

needed to eliminate the bacteria. We hypothesized that chronic Q fever patients have inadequate innate immune recognition of *C. burnetii*. In **Chapter 2**, we used functional *in-vitro* analysis to explore how *C. burnetii* is initially recognized by pattern-recognition receptors of the host immune cells. In **Chapter 3,** we investigated the association between chronic Q fever and genetic polymorphisms in these pattern-recognition receptors and their adaptor molecules. In **Chapter 4** we focussed on the IFN-γ response in chronic Q fever. IFN-γ is pivotal for microbicidal activity of monocytes and macrophages against the intracellular *C. burnetii*. Previous research has led to the conclusion that this pathway is defective in chronic Q fever patients. We started with a systems biology approach, using a gene expression microarray, to investigate the IFN-γ response in patients and healthy individuals. We investigated this further by functional and genetic analysis in chronic Q fever patients.

PART II. Detection of *C. burnetii* infection using cell-mediated immune responses

The standard immunological method to detect Q fever is measurement of specific antibodies against phase I and phase II *C. burnetii*. Assessment of T-lymphocyte immunity might be an additional and even superior method, given the fact that defence against *C. burnetii* mainly depends on cell-mediated immunity, which includes IFN-γ mediated macrophage activation. Hence, a skin test can be done to assess delayed-type hypersensitivity to intradermally injected inactivated *C. burnetii*. The skin test, however, is cumbersome, lacks a well-defined cut off, and depends on trained personnel to perform. Its use is limited to pre-vaccination screening to exclude potential vaccinees with pre-existing immunity against *C. burnetii*. We developed an in-vitro IFN-γ production assay, in which IFN-γ production is measured in blood after *in-vitro* exposure to *C. burnetii* antigens, using similar principles as those of the Quantiferon test for tuberculosis. In **Chapter 5,** we described how we investigated the applicability of our novel assay, by measuring the IFN-γ production in a large group of vaccination candidates who were screened by Q fever serology and skintest.

In **Chapter 6** we asked ourselves the very relevant question whether the measurement of IFN-γ production, in combination with other cytokines in a whole blood assay, could be used to differentiate an ongoing infection (chronic Q fever) from a past (cleared) infection. Identifying a cytokine profile specific for chronic Q fever may aid in its timely diagnosis and early treatment to prevent severe morbidity and death.

In **Chapter 7** we investigated whether cell-mediated immune responses can be used as a biomarker to monitor treatment of chronic Q fever. For this, we longitudinally performed whole blood assays in fifteen chronic Q fever patients on antibiotic treatment for at least 18 months.

Other IFN-γ release assays have recently been developed and are based on a technique of enzyme-linked immunosorbent spotting (Elispot), which detect antigen-specific IFN-γ secreting cells by making them visible at a single-cell level. In **Chapter 8**, we compared the results of our IFN-γ production assay with the results of a *Coxiella* Elispot in a group of Q fever patients and healthy control individuals.

In **Chapter 9** we asked the question whether the *C. burnetii* -specific IFN-γ responses could be a useful tool for diagnosis of acute Q fever. We challenged immunocompetent BALB/c mice with aerosols containing virulent phase I *C. burnetii*, and evaluated the timing and extent of IFN-γ responses. Other cytokines were also measured in an effort to identify other potential diagnostic markers.

PART III. Immunosuppressive drugs for rheumatoid arthritis as a risk factor for chronic Q fever

Although immune-suppression is a stated risk factor for development of chronic Q fever, based on case series and case reports, definition of the type of immune-suppression is poor and systematic studies are absent. Anti-TNF therapy increases the risk of infections with intracellular bacteria such as *M. tuberculosis*. Clinical data on the risk of *C. burnetii* infection in patients on anti-TNF therapy are lacking. In view of the widespread use of TNF-blockers in patients with rheumatoid arthritis (RA) or other inflammatory conditions, we decided to perform a controlled cohort study to examine the risk of Q fever in RA patients with and without anti-TNF therapy. **Chapter 10** describes the results of this study. In **Chapter 11** we described the history of an RA patient who had an episode of acute Q fever while being treated with anti-TNF agents, and who developed chronic Q fever over the subsequent two years while using the anti-B-cell monoclonal antibody rituximab. The case highlights the importance of cellular and humoral immune response modifying agents in the natural course of *C. burnetii* infections and the possible pitfalls of the use of serological methods to detect the stage of disease.

PART IV. Prevention of Q fever by vaccination against *C. burnetii*

Following the large Q fever outbreak in the Netherlands between 2007 and 2011, the Dutch government decided to vaccinate people with risk-conditions for development of chronic Q fever. The only available whole-cell vaccine Q-vax required pre-vaccination screening – skin-testing and serology – to exclude sensitized individuals with presumably more risk for immune-mediated adverse events following immunization. There was no experience worldwide with this vaccine in this specific old age risk group with its many co-morbidities and medication. Another special aspect of this vaccination campaign was that the IFN-γ release assay was performed during pre-vaccination screening. This

supplied additional information about pre-existing T-cell immunity in vaccinees as it did not influence the decision to vaccinate.

In **Chapter 12,** we describe the safety and adverse events after skin test and vaccination in this specific patient-group, and relate this to characteristics of the vaccinees and pre-vaccination screening outcomes. We also analyzed the association between local adverse events after skin test or vaccination, patient characteristics and pre- and post-vaccination immunological parameters. In **Chapter 13**, we assessed the immune responses to *C. burnetii* at 6 and 12 months after vaccination in this relevant population.

Chapter 14 focuses on the skin test which, as an *in-vivo* test, elicits an immune response itself and may influence subsequent measurements of immune response. In **Chapter 15**, we retrospectively aimed to obtain prevalence rates of the risk-conditions for chronic Q fever in the high-incidence area, in order to estimate coverage of the Q fever vaccination campaign. To this end, we extracted data on the specific risk-conditions from the Integrated Primary Care Information (IPCI)-database, a large longitudinal population-based general practice research database in the Netherlands. We described the procedure we followed to arrive at an estimate of the coverage of the Q fever vaccination campaign in the Netherlands 2011.

Finally, **Chapter 16** provides a summary, general discussion and recommendations for future research.

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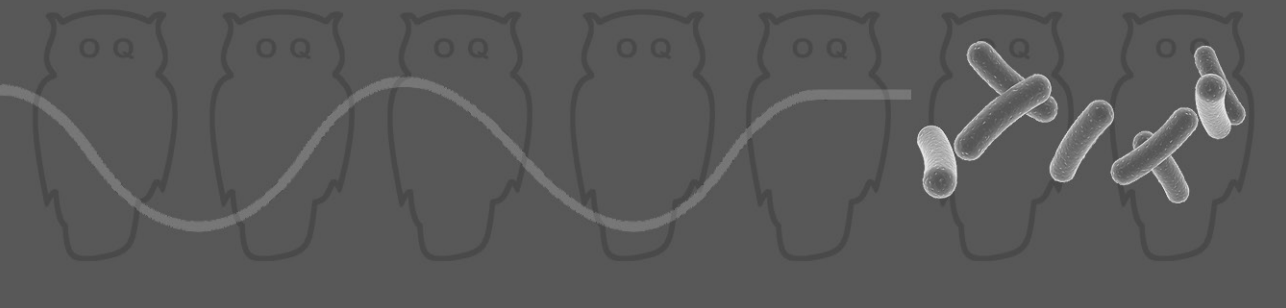
I

Studies on the immune response against *C. burnetii* in (chronic) Q fever



2

Recognition of *Coxiella burnetii* by Toll-like receptors and Nucleotide-binding oligomerization domain-like receptors

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Abstract

Background. Infection with *Coxiella burnetii* can lead to acute and chronic Q fever. Toll-like receptor 1 (TLR1), TLR2, TLR4, TLR6, nucleotide-binding oligomerization domain receptor 1 (NOD1), NOD2, and the mitogen-activated protein kinases are central in the innate immune response against microorganisms, but little is known about their role in the recognition of *C. burnetii* in humans.

Methods. Human peripheral blood mononuclear cells (PBMCs) were stimulated with *C. burnetii* Nine Mile and the Dutch outbreak isolate *C. burnetii* 3262. TLRs were inhibited using specific antibodies or antagonists. Additionally, the influence of human polymorphisms in TLRs and Nod-like receptors (NLRs) on *C. burnetii*–induced cytokine production was assessed.

Results. Inhibition of TLR2, p38, JNK, and ERK led to decreased cytokine responses in *C. burnetii*–stimulated human PBMCs. Humans with polymorphisms in *TLR1* and *NOD2* had reduced cytokine production, compared with humans with wild-type genotypes, after stimulation. Interestingly, polymorphisms in *TLR6* led to decreased cytokine production after *C. burnetii* 3262 stimulation but not after *C. burnetii* Nine Mile stimulation.

Conclusions. The TLR1/TLR2 heterodimer and NOD2 are important recognition receptors for the induction of cytokine responses against *C. burnetii* in humans. Furthermore, an interesting finding was the divergent recognition of *C. burnetii* Nine Mile and *C. burnetii* 3262.

Introduction

Coxiella burnetii is the causative agent of Q fever and can cause either acute or chronic infection in humans. Acute Q fever is associated with inflammation, hepatitis, and pneumonia. Chronic Q fever, defined as a cardiovascular chronic infection, develops over several months to years following an initial infection and is characterized primarily by endocarditis or endovascular infection [1]. Even though only a small proportion (2%–5%) of the infected individuals develop chronic Q fever, the burden for patients is significant, as long-term antimicrobial drug treatment is necessary, with mortality reaching >60% when infection is left untreated [1, 2]. In the Netherlands, one specific *C. burnetii* genotype, referred to as *C. burnetii* 3262, dominated the Q fever outbreak, which resulted in several thousands of cases and chronic infection in >250 patients [3].

The innate immune system provides the first line of host defense against microorganisms. Unique pathogen-associated molecular patterns (PAMPs) on the surfaces of microorganisms are recognized by the pattern-recognition receptors (PRRs) of the host immune cells to activate host-defense mechanisms, such as phagocytosis and cytokine signaling [4]. The main PRRs involved in recognition of bacteria are Toll-like receptors (TLRs; TLR1, TLR2, TLR4, and TLR6) and nucleotide-binding oligomerization domain receptor 1 (NOD1) and NOD2. Previous studies in mice and reporter cell lines have demonstrated TLR2 as an important regulator of immune responses against *C. burnetii* [5–7]. The TLR2 receptor is able to form heterodimers with either TLR1 or TLR6; TLR1/TLR2 heterodimers mainly recognize triacylated lipopeptides, whereas TLR2/TLR6 heterodimers recognize diacylated lipopeptides [8, 9]. Polymorphisms in *TLR1* and *TLR6* have been associated with increased susceptibility to candidemia, invasive aspergillosis, impaired mycobacterial signaling, and innate immune responses in sepsis [10–13]. NOD1 and NOD2 recognize the bacterial peptidoglycan structures g-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively [14]. They are associated with the recognition of a broad range of bacteria and interact with TLR2 through receptor-interacting protein (RIP)-like interacting caspase-like apoptosis regulatory protein kinase (RICK) as the central signaling molecule [15].

Two unknown issues involve how *C. burnetii* is initially recognized by the immune system in humans and how *C. burnetii* evades these mechanisms and leads to acute and chronic Q fever in patients. Previously, it has been shown that the defective immune response that accompanies chronic Q fever is associated with the failure of *C. burnetii* to induce lymphocyte proliferation, the absence of granulomas, and cytokine dysfunction [1, 16]. However, our group recently showed that patients with chronic Q fever have high T-cell–derived interferon γ (IFN-γ) production upon contact with *C. burnetii* [17]. This finding may indicate that

defective innate immune recognition of *C. burnetii* and subsequent induction of appropriate cytokines are important for the pathogenesis of chronic Q fever and may not be due to the failure to activate lymphocytes. To investigate this hypothesis, more fundamental knowledge is needed about the PRRs involved in sensing *C. burnetii*. Therefore, this study investigated the role of TLR1, TLR2, TLR4, TLR6, NOD1, NOD2, and the downstream mitogen-activated protein kinases (MAPKs) p38, JNK, and ERK in the recognition of two *C. burnetii* phase I strains, the reference strain *C. burnetii* Nine Mile and the Dutch outbreak isolate *C. burnetii* 3262. The study will provide better insight into the recognition of *C. burnetii* by PRRs in general and could assist further investigations on immunological causes of chronic Q fever.

Material and Methods

C. burnetii Strains

Two *C. burnetii* strains were used: *C. burnetii* Nine Mile RSA493 (NM) phase I (a kind gift from the Bundeswehr Institute for Microbiology; Munich, Germany) and *C. burnetii* X09003262 (3262) phase I, isolated from the placenta of an aborted goat in the Netherlands [3]. Both strains were cultured on buffalo green monkey cells, and the numbers of *Coxiella* DNA copies was determined using Taqman real-time polymerase chain reaction as described previously [18]. Lipopolysaccharide (LPS) phase determination was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and silver staining, using purified phase I (RSA493) and phase II (RSA439) *C. burnetii* NM LPS (kindly provided by R. Toman) as controls [19, 20]. Both *C. burnetii* strains were inactivated by heating for 30 minutes at 99°C.

Study Population

Peripheral blood mononuclear cells (PBMCs) were derived from buffy coats from blood donors (Sanquin, Nijmegen, the Netherlands) and from whole-blood specimens from volunteers (for single-nucleotide polymorphism [SNP] analyses). All were healthy Dutch volunteers of European descent. Donor blood specimens were not serologically screened for *C. burnetii* infection, and the individuals were not immunized [21]. In the SNP experiment with *C. burnetii* 3262, cells were isolated from 85 volunteers (age, 23–73 years; 23% were women, and 77% were men). In the experiment with *C. burnetii* NM, cells were isolated from 123 volunteers (age, 22–70 years; 16% were women, and 84% were men). The NOD2-deficient individuals had the homozygous NOD2 3020insC polymorphism. The 3020insC polymorphism was analyzed as described previously [22]. Venous blood specimens were collected after informed consent was obtained. The experiments were conducted according to the principles expressed in the Declaration of Helsinki.

Isolation of Human PBMCs and Stimulation

PBMCs from healthy and NOD2-deficient individuals were isolated as described previously [23]. A volume of 100 μL containing 5×10^5 cells was added to a round-bottomed 96-well plate (Corning, Amsterdam, the Netherlands) and incubated with *C. burnetii* NM or *C. burnetii* 3262 (range, $1 \times 10^6 - 1 \times 10^7$ bacteria/mL) in the presence of 10% human serum. After 24 hours, supernatants were harvested and stored at −20°C. In some experiments, PBMCs were preincubated for 60 minutes with antagonizing or inhibiting agents. Monoclonal antibodies for blocking TLR2 (clone TL2.1) and mouse immunoglobulin G2a isotype were used at a concentration of 5 μg/mL (InvivoGen, Toulouse, France). Highly purified *Bartonella quintana* LPS (100 ng/mL) was used as a TLR4 antagonist [24]. p38 was inhibited using 1 mmol/L p38 inhibitor SB202190 (Sigma-Aldrich, Zwijndrecht, the Netherlands) [25]. JNK and ERK1/2 were inhibited by 20 μmol/L JNK SP600125 (AG Scientific, San Diego, California) and 10 μmol/L MEK U0126 (Promega, Madison, Wisconsin), respectively.

Cytokine Measurement

Cytokine production was measured using an enzyme-linked immunosorbent assay (ELISA). The following kits were used: interleukin 6 (IL-6; Sanquin, Amsterdam, the Netherlands), tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β; R&D Systems, Minneapolis, Minnesota), and mouse IL-6 (Invitrogen, Carlsbad, California). Absorption was measured at 450 nm (Bio-Rad Laboratories, Veenendaal, the Netherlands).

Genomic DNA Isolation and SNP Analysis

DNA was isolated using the Gentra Pure Gene Blood kit (Qiagen, Valencia, California) according to the manufacturer's protocol for whole-blood specimens. Polymorphisms were identified using a predesigned TaqMan SNP genotyping assay (Applied Biosystems, Foster City, California) or an Illumina Immunochip. The following SNPs were analyzed: for *TLR1*: rs5743611, rs4833095, and rs5743618; for *TLR2*: rs5743704; for *TLR4*: rs4986790/rs4986791; for *TLR6* P249S: rs5743810; for *NOD2*: rs2066842, rs5743293, rs2066845, rs2066844, rs9302752, rs7194886, rs8057341, rs751271, and rs3135499; and *NOD1* insertion/deletion polymorphism *NOD1* + 32656 (Table 1).

Animals

TLR1, *TLR2*, and *TLR6* knockout mice were kindly provided by Prof S. Akira (Department of Host Defense, Osaka University, Osaka, Japan) and are fully backcrossed to the C57BL/6 background. Age- and sex-matched control C57BL/6 mice were obtained from Charles River Wiga (Sulzfeld, Germany). Mice were housed in filter-top cages, and water and food were provided ad libitum. Wild-type, *NOD1*, *NOD2*, and *NOD1*/2 knockout mice were bred and maintained in the St.

Table 1 Single-Nucleotide Polymorphisms (SNPs) Used in the Study

Gene	SNP	Mutation	Nucleotide Change[a]	Amino Acid Change	TaqManAssay
TLR1	rs4833095	Missense	C >T	S248N	C_44103606_10
TLR1	rs5743611	Missense	G >C	R80T	C_27855269_10
TLR1	rs5743618	. . .	G >T	S602I	Hs00248869_CE
TLR2	rs5743704	Missense	C >A	P631H	C_25607736_10
TLR4	rs4986790	Missense	A >G	D299G	C_11722238_20
TLR4	rs4986791	Missense	C >T	T399I	C_11722237_20
TLR6	rs5743810	Missense	C >T	P249S	C_1180648_20
NOD1	rs6958571[b]	. . .	. . .	+32656	. . .
NOD2	rs2066842	Missense	C >T	P268S	C_11717470_20
NOD2	rs2066847	Frameshift	. . .	1007finsC	. . .
NOD2	rs2066845	Missense	C >G	G908R	C_11717466_20
NOD2	rs2066844	Missense	C >T	R702W	C_11717468_20
NOD2	rs9302752	. . .	C >T	. . .	Genotyped using the Illumina Immunochip platform, as described by Smeekens et al [26]
NOD2	rs7194886	. . .	C >T	. . .	
NOD2	rs8057341	. . .	A >G	. . .	
NOD2	rs751271	. . .	G >T	. . .	
NOD2	rs3135499	. . .	A >C	. . .	

[a] The first nucleotide (and corresponding amino acid) is the ancestral nucleotide and therefore is considered the wild-type allele.

[b] This is the SNP database reference for a T/G single base pair substitution at the same position without the accompanying insertion/deletion.

Jude Children's Research Hospital (Memphis, Tennessee). Bone marrow–derived macrophages (BMDMs) were used in all experiments. After dissection of mouse legs, the bone marrow was flushed out using sterile phosphate-buffered saline (PBS). Differentiation into macrophages occurred in 7 days at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 30% L929 medium, 10% heatinactivated filtered fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 1% nonessential amino acids, and 1% 100 U/mL penicillin and 100 mg/mL streptomycin. On day 6, the BMDMs were counted and added to either a 24-well plate at a concentration of 1×10^6 cells/mL or a 96-well plate at a concentration of 1×10^5 cells/mL. After 24 hours of resting at 37°C, the cells were stimulated with medium, *C. burnetii* NM (1×10^6 bacteria/mL), *C. burnetii* 3262 (1×10^6–1×10^7 bacteria/mL), Pam3Cys (10 μg/ mL), and *Escherichia coli* LPS (10 ng/mL).

The experiments were approved by the Ethics Committee on Animal Experiments of the Radboud University Medical Center, and protocols were approved by the St. Jude Children's Research Hospital Committee on the Use and Care of Animals.

Statistical Analysis

The data are expressed as mean (± standard error of the mean) unless indicated otherwise. Differences between experimental groups were tested using the Mann–Whitney *U* test. In experiments using antagonists or inhibitors, the Wilcoxon matched-pairs signed rank test was used. GraphPad Prism 5 software was used. Differences with a P value of $< .05$ were considered statistically significant.

Results

Role for TLR2, but not TLR4, in *C. burnetii*–Induced Cytokine Response in Humans

TLR4 is an important receptor for LPS. Because *C. burnetii* phase I possesses tetra-acylated LPS [27], TLR4 could play a role in cytokine production by *C. burnetii*. Upon TLR4 blockade by the TLR4 antagonist *Bartonella* LPS, the IL-1β, IL-6, and TNF-α responses of PBMCs after *E. coli* LPS stimulation were effectively inhibited. However, this potent TLR4 antagonist was unable to inhibit *C. burnetii* NM–induced and *C. burnetii* 3262–induced cytokine responses in PBMCs (Figure 1*A*).

PBMCs preincubated with an antagonistic antibody directed against TLR2 produced less IL-1β and IL-6 after encounter with *C. burnetii* NM and *C. burnetii* 3262 (Figure 1*B*). TNF-α levels were decreased after stimulation with *C. burnetii* 3262; conversely, this was not observed after stimulation with *C. burnetii* NM and the TLR2 ligand Pam3Cys (Figure 1*B*). Additionally, the role of TLR4 and TLR2 in

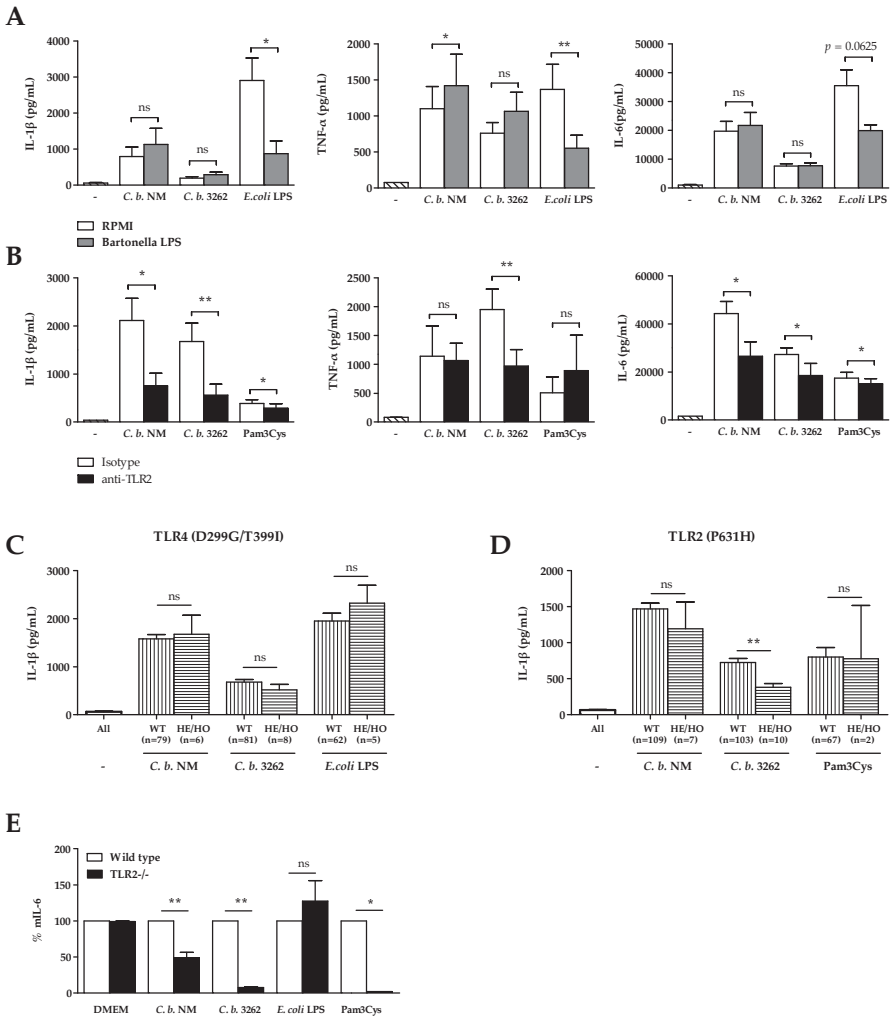

Figure 1. Recognition of *Coxiella burnetii* by Toll-like receptor 2 (TLR2), but not by TLR4, in humans and mice. *A* and *B*, Peripheral blood mononuclear cells (PBMCs) were pretreated with medium or *Bartonella* lipopolysaccharide (LPS; 100 ng/mL; *A*) or with isotype control immunoglobulin G2B or anti-TLR2 (*B*) for 1 hour and subsequently stimulated with Roswell Park Memorial Institute (RPMI) medium (striped bar), *C. burnetii* Nine Mile (NM; 1×10^6 bacteria/mL), *C. burnetii* 3262 (1×10^6 bacteria/mL), *Escherichia coli* LPS (10 ng/mL), or Pam3Cys (10 μg/mL) in the presence of serum (n = 5–9). Mean values (± standard error of the mean [SEM]) are shown. **P* < .05 and ***P* < .01, by the 2-tailed Wilcoxon matched-pairs signed rank test. *C* and *D*, PBMCs from healthy individuals genotyped for the TLR4 D299G/T399I single-nucleotide polymorphism (*C*) and TLR2 P631H (*D*) were stimulated with RPMI medium (striped bar), *C. burnetii* NM (3×10^6 bacteria/mL), and *C. burnetii* 3262 (3×10^6 bacteria/mL) in the presence of serum. Data are mean values (±SEM). **P* < .05 and ***P* < .01,

by the Mann–Whitney *U* test. Interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), and interleukin 6 (IL-6) were measured in the supernatant by an enzyme-linked immunosorbent assay (ELISA) after 24 hours of incubation. *E*, Bone marrow–derived macrophages from wild-type (WT) and TLR2 knockout mice were stimulated with Dulbecco's modified Eagle's medium, *C. burnetii* NM (1×10^6 bacteria/mL), *C. burnetii* 3262 (1×10^6 bacteria/mL), Pam3Cys (10 μg/mL), and *E. coli* LPS (10 ng/mL). The murine IL-6 (mIL-6) level was measured using ELISA after 24 hours of incubation. Data are combined results of 2 separate experiments containing 3 mice in each group (Pam3Cys stimulation, 1 experiment only). The mIL-6 production of the WT mice is set at 100%. Abbreviations: HE, heterozygote; HO, homozygote; NS, not significant.

C. burnetii recognition was investigated using PBMCs from volunteers with or without SNPs in these genes. Polymorphisms in *TLR4* (D299G and T399I) did not result in decreased cytokine responses (Figure 1*C*). PBMCs from volunteers homozygous for a SNP in *TLR2* (P631H) showed significantly decreased IL-1β responses after stimulation with *C. burnetii* 3262 only (Figure 1*D*). An explanation can be that the location of the TLR2 SNP is more important for the binding of *C. burnetii* 3262 than for *C. burnetii* NM or Pam3Cys.

Finally, we investigated the role of TLR2 in the cytokine induction by *C. burnetii* in murine BMDMs. BMDMs derived from TLR2 knockout mice produced less IL-6, compared with those from wild-type mice, after stimulation with *C. burnetii* NM and *C. burnetii* 3262 (Figure 1*E*).

Cytokine Induction by *C. burnetii* NM Stimulation of Human PBMCs Depends on TLR1, While *C. burnetii* 3262 Recognition Is Mediated by Both TLR1 and TLR6

The finding that TLR2 plays an important role in the recognition of *C. burnetii* led to the question whether this response is mainly mediated by TLR1/TLR2 or TLR2/TLR6 heterodimers [28].

PBMCs from individuals bearing the homozygous mutant variant of *TLR1* (S609I) and *TLR1* (N248S) produced significantly less IL-1β and TNF-α after stimulation with *C. burnetii* NM and *C. burnetii* 3262 than individuals who were heterozygous for these particular SNPs or had the wild-type variant (Figure 2*A*). The same trend was observed for the *TLR1* (R80T) SNP, of which only a few individuals in the cohort carried the homozygous variant. Decreased cytokine responses were also observed in these individuals after stimulation with the purified TLR2 ligand Pam3Cys (data not shown). Among the individuals carrying the *TLR6* (P249S) SNP, the cytokine responses induced by the two *C. burnetii* strains differed from each other. *C. burnetii* NM stimulation did not result in decreased IL-1β and TNF-α production, while stimulation with *C. burnetii* 3262 revealed significantly lower IL-1β and TNF-α production, compared with individuals who were heterozygous or had the wild-type variant (Figure 2*B*). However, stimulation

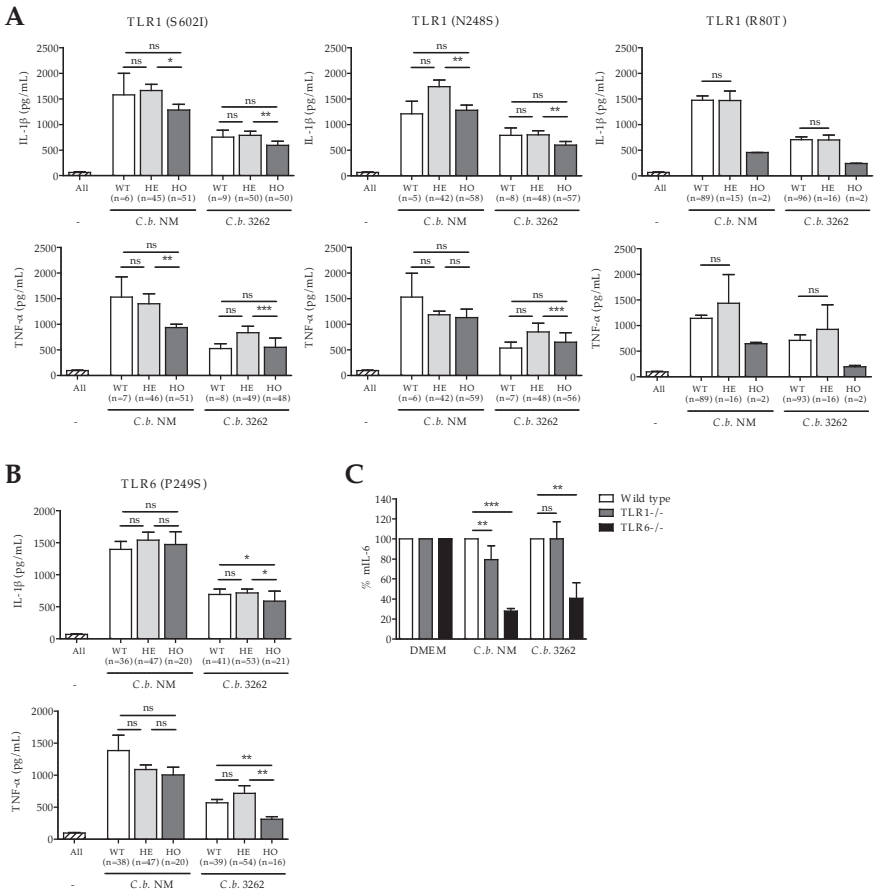

Figure 2. Recognition of *Coxiella burnetii* by Toll-like receptor 1 (TLR1) and TLR6 in humans and mice. *A* and *B*, Peripheral blood mononuclear cells from healthy individuals genotyped for the TLR1 S602I, TLR1 N248S, and TLR1 R80T single-nucleotide polymorphisms (SNPs; *A*) and the TLR6 P249S SNP (*B*) were stimulated with Roswell Park Memorial Institute medium (RPMI) (striped bar), *C. burnetii* Nine Mile (NM; 3×10^6 bacteria/mL), and *C. burnetii* 3262 (3×10^6 bacteria/mL) in the presence of serum. Interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) levels were measured in the supernatant by an enzyme-linked immunosorbent assay (ELISA) after 24 hours of incubation. **P* < .05, ***P* < .01, and ****P* < .001, by the Mann–Whitney *U* test. *C*, Bone marrow–derived macrophages from wild-type (WT), TLR1 knockout, and TLR6 knockout mice were stimulated with medium only, *C. burnetii* NM (1×10^6 bacteria/mL), and *C. burnetii* 3262 (1×10^6–1×10^7 bacteria/mL). After 24 hours of stimulation, mouse IL-6 levels were measured by ELISA. White bars represent the WT mice, gray bars represent the TLR1 knockout mice, and black bars represent the TLR6 knockout mice. Experiments were performed in duplicate (3–4 mice in each group), and mean values (± standard error of the mean) are depicted. IL-6 production by the WT mice is set at 100%. **P* < .05 and ***P* < .01, by the Mann–Whitney *U* test. Abbreviations: DMEM, Dulbecco's modified Eagle's medium; HE, heterozygote; HO, homozygote; mIL-6, mouse interleukin 6.

with fibroblast stimulating ligand 1 (FSL-1), a TLR2/6 agonist, did not result in lowered cytokine responses (data not shown).

In mice, BMDMs derived from TLR1 knockout mice produced less IL-6 after stimulation with *C. burnetii* NM, while this was not observed after *C. burnetii* 3262 stimulation. On the other hand, IL-6 production by BMDMs from TLR6 knockout mice was strongly diminished after stimulation with both *C. burnetii* NM and *C. burnetii* 3262 (Figure 2*C*).

The Role of NOD2 in the *C. burnetii*–Induced Cytokine Response

NLRs are involved in initiating an immune response against microorganisms through recognition of bacterial peptidoglycan. As shown in Figure 3*A*, PBMCs from individuals heterozygous and homozygous for the NOD1 insertion-deletion + 32656 polymorphism displayed similar IL-1β and TNF-α production after *C. burnetii* NM and *C. burnetii* 3262 stimulation as individuals bearing the wild-type genotype.

In contrast, PBMCs from NOD2-deficient individuals produced significantly less IL-1β and IL-6 than PBMCs from healthy controls after *C. burnetii* 3262 stimulation (Figure 3*B*). The same trend was observed after *C. burnetii* NM stimulation (Figure 3*B*) and for TNF-α production (data not shown). The effect of 9 NOD2 SNPs was further analyzed. Stimulation with *C. burnetii* NM resulted in decreased yet not statistically significant TNF-α production in 6 of these 9 analyzed NOD2 SNPs, while 4 of these SNPs modulated stimulation with *C. burnetii* 3262, resulting in lower TNF-α and IL-1β production. Figure 3*C* shows these TNF-α responses of the NOD2 1007finsC, NOD2 G908R, and NOD2 R702W SNPs after *C. burnetii* NM and *C. burnetii* 3262 stimulation.

In mice, *C. burnetii* NM stimulation of BMDMs led to a 35% reduction of IL-6 production in NOD1 knockout mice, compared with wild-type mice, while in NOD2 knockout mice and double-knockout mice, IL-6 production was reduced by >50% (Figure 3*D*). Unfortunately, stimulation of the BMDMs with *C. burnetii* 3262 resulted in low production of IL-6 and was not useful for comparing the different groups (data not shown).

P38, JNK, and ERK Are Downstream Mediators of *C. burnetii*–Induced Cytokine Production

MAPKs such as p38, JNK, and ERK have been proven to be downstream of TLRs, and their activation eventually leads to cytokine production, proliferation, and apoptosis [29]. We confirmed this function of MAPKs upon *C. burnetii* stimulation, as inhibition of p38 and ERK led to significantly decreased IL-1β, TNF-α, and IL-6 production by PBMCs (Figure 4). PBMCs preincubated with a specific JNK inhibitor also produced less IL-1β and TNF-α after *C. burnetii* stimulation, but IL-6 production was not altered in comparison to the dimethyl sulfoxide control (Figure 4).

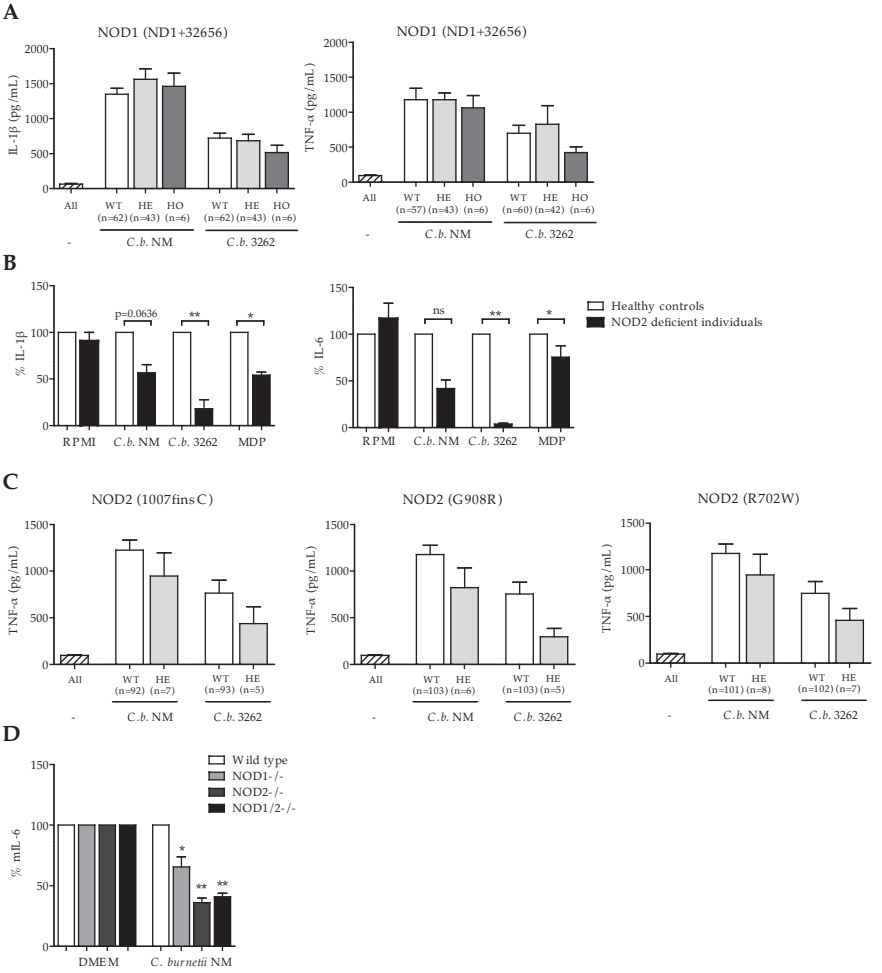

Figure 3. Nucleotide-binding oligomerization domain receptor 2 (NOD2) is involved in the recognition of *Coxiella burnetii*, while NOD1 is not. *A*, Peripheral blood mononuclear cells (PBMCs) from healthy volunteers genotyped for the NOD1 single-nucleotide polymorphism (SNP) ND1 + 32656 were stimulated with Roswell Park Memorial Institute (RPMI) medium (striped bar), *C. burnetii* Nine Mile (NM; 3 × 10^6 bacteria/mL), and *C. burnetii* 3262 (3 × 10^6 bacteria/mL). *B*, PBMCs from individuals with a homozygous NOD2 3020insC mutation and PBMCs from healthy controls were stimulated with RPMI medium, *C. burnetii* NM (1× 10^6–2× 10^6 bacteria/mL), *C. burnetii* 3262 (1× 10^6–2× 10^6 bacteria/mL), and muramyl dipeptide (MDP; 5 μg/mL). Interleukin 1β (IL-1β) and interleukin 6 (IL-6) production by the healthy controls is set at 100%. *C*, PBMCs from healthy volunteers genotyped for the NOD2 SNPs 1007finsC, NOD2 G908R, and NOD2 R702W were stimulated with RPMI medium (striped bar), *C. burnetii* NM (3 × 10^6 bacteria/mL), and *C. burnetii* 3262 (3 × 10^6 bacteria/mL). In all experiments, PBMCs were stimulated in the presence of human serum. Interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α) levels were measured in the supernatant by an

enzyme-linked immunosorbent assay (ELISA) after 24 hours of incubation. **P* < .05 and ***P* < .01, by the Mann–Whitney *U* test. *D*, Bone marrow–derived macrophages from wild-type (WT), NOD1 knockout, NOD2 knockout, and NOD1/2 knockout mice were stimulated with medium only and *C. burnetii* NM (1×10^7 bacteria/mL). After 24 hours of stimulation, mouse IL-6 levels were measured by ELISA. White bars represent the WT mice, light gray bars represent the NOD1 knockout mice, dark gray bars represent the NOD2 knockout mice, and black bars represent the NOD1/2 knockout mice. Experiments were performed in duplicate (n = 2–3), and mean values (± standard error of the mean) are depicted. IL-6 production by the WT mice is set at 100%. **P* < .05 and ***P* < .01, by the Mann–Whitney *U* test. Abbreviations: DMEM, Dulbecco's modified Eagle's medium; HE, heterozygote.

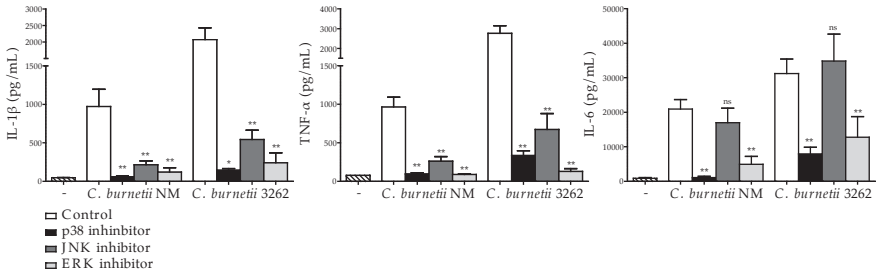

Figure 4. Involvement of mitogen-activated protein kinases in *Coxiella burnetii*–induced cytokine production. Peripheral blood mononuclear cells from 8 healthy volunteers were pretreated with dimethyl sulfoxide (control), p38 inhibitor, JNK inhibitor, or ERK inhibitor for 1 hour and subsequently stimulated with Roswell Park Memorial Institute (RPMI) medium (striped bar), *C. burnetii* Nine Mile (NM; 1×10^6 bacteria/mL), and *C. burnetii* 3262 (1×10^6 bacteria/ mL) in the presence of human serum. Interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), and interleukin 6 (IL-6) were measured in the supernatant by an enzyme-linked immunosorbent assay after 24 hours of incubation. Mean values (± standard error of the mean) of 3 separate experiments are shown. **P* < .05 and ***P* < .01, by the 2-tailed Wilcoxon matched-pairs signed rank test.

Discussion

In the present study, we investigated the role of TLRs and NLRs in the recognition of *C. burnetii*. Using different approaches, we found clear evidence for involvement of TLR1, TLR2, and NOD2 but not for TLR4 and NOD1. An additional important finding was the divergent recognition of *C. burnetii* NM and the Dutch outbreak isolate *C. burnetii* 3262. *C. burnetii* NM was mediated by TLR1/TLR2 only, while *C. burnetii* 3262 was recognized by both the TLR1/TLR2 and TLR2/TLR6 heterodimers. Interestingly, recognition of these two strains also differed with respect to the role of TLR6 in men and mice.

We found no important role of TLR4 in mediating in vitro cytokine production by human PBMCs after *C. burnetii* encounter. Although Honstrette et al [30] reported that TLR4 knockout mice exhibited a defect in cytokine production and granuloma formation, our observation that *C. burnetii*–induced cytokine production in humans was not mediated by TLR4 is not surprising. As reported before, LPS with a tetra-acylated lipid A component, as expressed by bacteria such as *Rhodobacter sphaeroides* or the lpxL2 mutant of *Neisseria meningitidis* H44/76, is found to be a very weak cytokine inducer and even to act as a TLR4 antagonist [31, 32]. Accordingly, an antagonistic effect of *C. burnetii* phase I lipid A has been demonstrated [7].

We observed an important role of TLR2 in *C. burnetii* recognition in mice, and, moreover, our study revealed that TLR2 is also important for *C. burnetii* recognition by human PBMCs. *C. burnetii* NM and *C. burnetii* 3262 are both able to induce cytokine production via TLR2 signaling, which suggests that both strains have PAMPs that can activate TLR2. *C. burnetii* phase II activation of TLR2 has previously been reported by Zamboni et al [7]. They showed that macrophages from TLR2 knockout mice were highly permissive to *C. burnetii* growth, compared with either TLR4 knockout or wild-type mice. Furthermore, they found that macrophages from TLR2 knockout mice produced lower levels of TNF-α and IL-12 [7]. While Zamboni et al used the avirulent *C. burnetii* phase II, we used the virulent *C. burnetii* phase I, which is the infective phase that is found in the environment.

We additionally investigated the role of TLR1 and TLR6 in cytokine induction by *C. burnetii*, because TLR2 forms heterodimers with either TLR1 or TLR6. We addressed the issue by performing experiments with PBMCs from individuals with known SNPs in *TLR1* and *TLR6*. We found that individuals carrying polymorphisms in *TLR1* showed a diminished cytokine response against *C. burnetii* NM and *C. burnetii* 3262. Our findings suggest that the TLR1/TLR2 heterodimer plays a role in *C. burnetii* recognition. BMDMs from TLR1 knockout mice stimulated with *C. burnetii* NM produced less IL-6, compared with BMDMs from wild-type mice. Conversely, stimulation with *C. burnetii* 3262 did not lead to lower cytokine production in TLR1 knockout BMDMs.

The role of TLR6 in the induction of a cytokine response against *C. burnetii* is less clear. Our analyses of human PBMCs suggested that *C. burnetii* 3262 is recognized by TLR6, while this is not the case for *C. burnetii* NM. This can be explained by a different binding site of *C. burnetii* 3262 to TLR6, compared with *C. burnetii* NM and FSL-1. In mice, it seemed that TLR6 plays a major role in cytokine induction by *C. burnetii* for both the reference strain and the Dutch outbreak isolate. We observed a different role for TLR1 and TLR6 in mice and humans in our experiments, showing that innate immune recognition of *C. burnetii* can differ between mice and humans. However, much of the data published to this point on

innate immune recognition of *C. burnetii* is derived from murine experiments. We can conclude that assumptions about immune recognition of *C. burnetii* in humans based on murine studies alone should be made carefully [33, 34].

The shared intracellular localization of the NOD2 receptor and *C. burnetii*, together with the confirmed role of TLR2 in the recognition of *C. burnetii*, made the investigation of NOD2 of interest. Our study in humans revealed that NOD2 plays a major role in cytokine induction upon *C. burnetii* encounter, as individuals bearing the NOD2 3020insC mutation (which causes a loss of function of the NOD2 gene [22]) had significant lower cytokine responses. This finding was further strengthened by our observation in individuals bearing SNPs in *NOD2*. The important role for both TLR2 and NOD2 signaling in the induction of cytokines has been demonstrated for other bacteria, such as *Borrelia burgdorferi* and *Mycobacterium paratuberculosis* [23, 35]. So far, only one other research group has investigated the role of NOD2 in *C. burnetii* infection [36]. They concluded that NOD2 is not essential for the control of *C. burnetii* infection in mice, although they observed lower TNF-α and MCP-1 messenger RNA responses in *C. burnetii*–stimulated BMDMs from NOD2 knockout mice, compared with those from wild-type mice. We found lower IL-6 responses in *C. burnetii*–stimulated BMDMs from NOD1 knockout mice, compared with those for wild-type mice, but even a larger decrease was observed in NOD2 knockout mice. As the IL-6 response was not further decreased in the NOD1/2 knockout mice, we suggest that the role of NOD2 is more important.

The strain *C. burnetii* 3262 was the predominant genotype found during the Q fever outbreak in the Netherlands. This single genotype of *C. burnetii* caused the largest outbreak of Q fever ever reported worldwide. Besides increased exposure of humans to this pathogen due to intensive goat herding, it may be hypothesized that this strain is less effectively recognized by the human immune system and is therefore more virulent than other strains. However, our study showed that, similar to *C. burnetii* NM, *C. burnetii* 3262 in humans was recognized by TLR1, TLR2, and NOD2 and induced a robust proinflammatory cytokine response. Interestingly, we observed a possible role for TLR6 in cytokine induction by *C. burnetii* 3262, while this was not seen for *C. burnetii* NM. It can be questioned whether extended activation of the immune response is beneficial for the infected individual, as enhanced uptake of *C. burnetii* by monocytes and macrophages can favor the intracellular replication of *C. burnetii*, leading to a possible infection. However, it has to be further investigated whether the recognition of *C. burnetii* 3262 by TLR6 can lead to increased virulence.

It is not completely clear why chronic Q fever only develops in a small proportion of infected individuals. Although patient-related factors such as the presence of valvular pathology or atherosclerotic vascular disease are a

contributing factor, poly-morphisms in PRR genes may also predispose to the development of chronic Q fever. Studies performed in our group show associations between polymorphisms in *TLR1*, *TLR2*, and *TLR6* and increased susceptibility to complicated skin and skin structure infections [37]. In addition, others observed associations between the TLR6 SNP and malaria and between invasive aspergillosis and overrepresentation of TLR2 in patients with tuberculosis [13, 38, 39]. Therefore, it would be interesting for future studies to investigate whether polymorphisms in *TLR1*, *TLR2*, *TLR6*, and *NOD2* are observed more often in patients with chronic Q fever and whether these polymorphisms are related to the severity of infection due to any *C. burnetii* strain or to specific *C. burnetii* strains.

Notes

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3

Genetic variation in pattern recognition receptors and adaptor proteins associated with development of chronic Q fever

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Abstract

Background. Q fever is an infection caused by *Coxiella burnetii*. Persistent infection (chronic Q fever) develops in 1%–5% of patients. We hypothesize that inefficient recognition of *C. burnetii* and/or activation of host-defense in individuals carrying genetic variants in pattern recognition receptors or adaptors would result in an increased likelihood to develop chronic Q fever.

Methods. Twenty-four single-nucleotide polymorphisms in genes encoding Toll-like receptors, nucleotide-binding oligomerization domain–like receptor-2, $\alpha_v\beta_3$ integrin, CR3, and adaptors myeloid differentiation primary response protein 88 (MyD88), and Toll interleukin 1 receptor domain-containing adaptor protein (TIRAP) were genotyped in 139 patients with chronic Q fever and in 220 controls with cardiovascular risk-factors and previous exposure to *C. burnetii*. Associations between these single-nucleotide polymorphisms and chronic Q fever were assessed by means of univariate logistic regression models. Cytokine production in whole-blood stimulation assays was correlated with relevant genotypes.

Results. Polymorphisms in *TLR1* (R80T), *NOD2* (1007fsX1), and *MYD88* (–938C>A) were associated with chronic Q fever. No association was observed for polymorphisms in *TLR2*, *TLR4*, *TLR6*, *TLR8*, *ITGAV*, *ITGB3*, *ITGAM*, and *TIRAP*. No correction for multiple testing was performed because only genes with a known role in initial recognition of *C. burnetii* were included. In the whole-blood assays, individuals carrying the *TLR1* 80R-allele showed increased interleukin 10 production with *C. burnetii* exposure.

Conclusions. Polymorphisms in *TLR1* (R80T), *NOD2* (L1007fsX1), and *MYD88* (–938C>A) are associated with predisposition to development of chronic Q fever. For *TLR1*, increased interleukin 10 responses to *C. burnetii* in individuals carrying the risk allele may contribute to the increased risk of chronic Q fever.

Introduction

Q fever is an infection with the intracellular gramnegative bacterium *Coxiella burnetii*. Although most symptomatic individuals experience only mild flulike symptoms or pneumonia (acute Q fever), some patients develop a persistent infection with severe complications including endocarditis and vascular (prosthesis) infection (chronic Q fever). Persistent infection occurs in 1%–5% of *C. burnetii*–infected subjects and develops insidiously, which contributes to its late diagnosis and high mortality rates. It is well established that the main risk factors for chronic infection are preexisting abnormalities of cardiac valves (including valvular prosthesis), vascular aneurysms, vascular prosthesis, and immunosuppression [1, 2]. However, in a large outbreak in the Netherlands from 2007 to 2010, only a minority of patients with these risk factors developed chronic Q fever after (serological evidence of) infection with *C. burnetii* [3, 4]. Inefficient early recognition of the bacterium by the innate immune system followed by incomplete eradication and/or inadequate initiation of adaptive immune responses may be a contributing factor in the development of chronic Q fever.

The innate immune system provides the first barrier against *C. burnetii* infection. In general, pattern recognition receptors (PRRs) on innate immune cells recognize molecular moieties of microorganisms. Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) proteins are the main PRRs involved in the recognition of bacteria. TLRs interact with various adaptor proteins, including myeloid differentiation primary response protein 88 (MyD88) and Toll interleukin 1 receptor domaincontaining adaptor protein (TIRAP), to activate transcription factors, leading to production of proinflammatory cytokines, activation of antimicrobial mechanisms, and subsequent initiation of adaptive immune responses. MyD88 is also required for the production of interleukin 1, 18, and 33, which emphasizes its importance for host defense against microorganisms.

We recently demonstrated that *C. burnetii*–induced cytokine production by human innate immune cells is mediated through the heterodimer TLR1/TLR2 on the cell membrane and by cytoplasmic NOD2 [5]. TLR6 seems to be involved specifically in the recognition of the *C. burnetii* strain 3262 that was isolated during the outbreak in the Netherlands [5]. Microorganisms such as *C. burnetii* use phagocytosis as an efficient mechanism of entry into monocytes/macrophages, where they can survive intracellularly in a parasitophorous vacuole (PV) [6]. An important role in *C. burnetii* uptake has been described for leukocyte response integrin ($\alpha_v\beta_3$ integrin) and complement receptor 3 (CR3 [$\alpha M\beta 2$, CD11b/CD18]). Virulent phase I *C. burnetii* uptake is mediated by $\alpha_v\beta_3$ integrin, whereas phagocytosis of avirulent phase II *C. burnetii* also involves CR3 [7, 8].

Genetic variants in immune cell receptors such as TLRs and NODs and their adaptors have been associated with increased susceptibility to bacterial infections

[9–16]. Thus, it is tempting to speculate that inefficient recognition of *C. burnetii* and activation of host defense in individuals carrying genetic variants in these receptors or adaptors would result in ineffective early clearance of the bacterium and an increased likelihood to develop chronic Q fever. No study so far has been able to investigate this possibility, due to the low prevalence of the disease. The recent Q fever outbreak in the Netherlands led to >4000 reported acute Q fever cases and >250 chronic Q fever cases [17], offering an unique opportunity to study this question.

We hypothesized that certain polymorphisms in the host's PRR genes or in genes encoding their adaptor proteins influence the risk of persistent *C. burnetii* infection and hence the development of chronic Q fever. To test this hypothesis, we analyzed the prevalence of specific single-nucleotide polymorphisms (SNPs) in 11 candidate genes —*TLR1, TLR2, TLR4, TLR6, TLR8*, *MYD88*, *TIRAP, NOD2*, *ITGAV, ITGB3,* and *ITGAM* (*CD11b)*— in a cohort of 139 patients with chronic Q fever and a control cohort of 220 individuals, living in the same area and with valvular or vascular predisposing factors for chronic Q fever, who had contracted *C. burnetii* (based on positive serological results) but did not develop chronic Q fever. The genetic study was complemented with functional assays investigating the effect of the specific polymorphisms on the in vitro cytokine production in *C. burnetii*–stimulated whole blood.

Material and Methods

Ethics Statement

The study was approved by the Ethical Committee of Radboud University Medical Center, Nijmegen, the Netherlands. Subjects were enrolled after providing written informed consent (or waiver when deceased [n = 5], as approved by the Ethical Committee). Institutional review boards of participating hospitals approved the inclusion of patients and controls in this study. The study has been performed in accordance with the Declaration of Helsinki.

Subjects

All patients who visited the outpatient clinic at the internal medicine departments of the participating hospitals with probable or proven chronic Q fever, as defined by the Dutch consensus on chronic Q fever (Table 1), were asked to participate in the study. Recruitment of patients took place between July 2011 and July 2014 at Radboud University Medical Center, Canisius-Wilhelmina Hospital (Nijmegen), Catharina Hospital (Eindhoven), Elkerliek Hospital (Helmond), Atrium Medical Center (Heerlen), Elisabeth Hospital (Tilburg), Bernhoven Hospital (Oss), and Jeroen Bosch Hospital ('s-Hertogenbosch). Demographic and clinical characteristics

Table 1 Classification of Chronic Q Fever According to the Dutch Consensus Guidelines[a]

Classification	Criteria
Proven chronic Q fever	1. Positive *Coxiella burnetii* PCR findings in blood or tissue[b] OR 2. IFA titer ≥1:800 or ≥1:1024 for *C. burnetii* phase I IgG[c] AND – definite endocarditis according to modified Duke criteria [18] OR – proven large vessel or prosthetic infection shown by imaging studies (FDG-PET, CT, MR imaging, or AUS)
Probable chronic Q fever	IFA titer ≥1:800 or ≥1:1024 for *C. burnetii* phase I IgG[c] AND ≥1 of the following: – Valvulopathy not meeting major criteria of modified Duke criteria [18] – Known aneurysm and/or vascular or cardiac valve prosthesis without signs of infection shown by TEE/TTE, FDG-PET, CT, MR imaging, or AUS – Suspected osteomyelitis, pericarditis, or hepatitis as manifestation of chronic Q fever – Pregnancy – Symptoms and signs of chronic infection (e.g., as fever, weight loss and night sweats, hepatosplenomegaly, or persistently raised ESR and CRP level) – Granulomatous tissue inflammation, proved histologically – Immunocompromised state
Possible chronic Q fever	IFA titer ≥1:800 or ≥1:1024 for C. burnetii phase I IgG[c] without manifestations meeting criteria for proven or probable chronic Q fever

Abbreviations: AUS, abdominal ultrasound; CRP, C-reactive protein; CT, computed tomography; ESR, erythrocyte sedimentation rate; FDG-PET, fluorodeoxyglucose positron emission tomography; IFA, immunofluorescence assay; IgG, immunoglobulin G; MR, magnetic resonance; PCR, polymerase chain reaction; TEE, transesophageal echocardiography; TTE, transthoracic echocardiography.

[a] The consensus guidelines are described by Wegdam-Blans et al [19].

[b] In the absence of acute infection.

[c] Cutoff is dependent on the IFA technique used (≥1:800 for a technique developed in house and ≥1:1024 for a commercial IFA technique).

(classification based on serological titers and imaging results, cardiovascular risk factors, immunosuppressive comorbid condition, or medication) were retrieved from the patients' medical records.

The population-matched control group consisted of individuals from the same area who had valvular or vascular abnormalities predisposing to chronic

Q fever and serological evidence of exposure to *C. burnetii* (anti–*C. burnetii* phase II immunoglobulin G antibody titer ≥1:32) but no clinical symptoms or serological evidence of chronic Q fever. These individuals were recruited in the Q fever prevaccination screening campaign in January– April 2011 [20, 21] and in a Q fever screening of patients with vascular risk factors living in the Q fever outbreak area [22].

Genotyping

From patients who came to the outpatient clinic or to the Q fever prevaccination screening, venous blood was collected and stored at −80°C. DNA was isolated from these blood samples using standard methods [23]. Other participants, both patients and controls, received a buccal swab kit (Isohelix) to obtain epithelial cells for DNA isolation. DNA was isolated using a buccal DNA isolation kit (Isohelix), according to the manufacturer's protocol. SNPs were selected based on known effects on protein function or gene expression, published associations with human diseases, and/or haploview data. In total, 25 SNPs in *TLR1*, *TLR2*, *TLR4*, *TLR6*, *TLR8*, *TIRAP*, *MYD88*, *NOD2*, *ITGAV*, *ITGB3,* and *ITGAM* were genotyped with a Sequenom mass spectrometry genotyping platform. Quality control was performed by duplicating 5% of the samples within and across plates, incorporating positive and negative control samples, and sequencing samples to verify the various genotypes.

In Vitro Whole-Blood Stimulation and Cytokine Measurements

In a subgroup of control subjects—those who participated in the Q fever prevaccination screening study, as described elsewhere [24] —whole-blood assays were performed, and *C. burnetii*–induced cytokines were measured (n = 93). In short, venous blood was drawn into endotoxin-free lithium-heparin tubes (Vacutainer; BD Biosciences), and samples were processed within 12 hours. Blood was aliquoted in separate tubes and incubated at 37°C for 24 hours with heat-inactivated (30 minutes at 99°C) *C. burnetii* Nine Mile RSA493 phase I [25] (bacteria count, 10^7/mL) or mitogen (positive control) or without stimulus (negative control), as described elsewhere [24]. After incubation, blood cultures were centrifuged at 4656 *g* for 10 minutes, and supernatants were collected. Supernatants were stored at –20°C until concentrations of cytokines—including interleukin 1β (IL-1β), tumor necrosis factor (TNF), interleukin 2 (IL-2), interleukin 6 (IL-6), and interleukin 10 (IL-10)—were measured with a magnetic bead multiplex assay (Merck Millipore), according to the manufacturer's instructions.

Statistical Analyses

The presence of Hardy–Weinberg equilibrium was analyzed for all 24 SNPs separately in the control cohort [26]. For the 2 TLR8 SNPs on the X chromosome,

only the female subjects were included in the Hardy–Weinberg equilibrium analysis, and relative allele frequencies were subsequently compared between female and male subjects. The difference in genotype frequencies between patients and controls were analyzed by means of a gene dosage model, using the Fisher exact test to test for significance. Subsequent dominant and recessive model analysis was performed using univariate logistic regression, for which odds ratios (ORs) and their 95% confidence intervals (CIs) were reported. Because the choice of the genetic variants was based exclusively on genes with an established or suspected role in *C. burnetii* recognition, rather than exploratory, no correction for multiple testing was performed. Statistical analyses were performed with SPSS software (version 20). Cytokine concentrations from the whole-blood stimulation assays were stratified according to genotype and compared by means of Mann–Whitney *U* tests, using GraphPad Prism software (version 5; GraphPad). Overall, statistical tests were 2 sided, and differences were considered statistically significant at $P < .05$.

Results

Baseline Characteristics

In total, 139 (92 proved and 47 probable) patients with chronic Q fever and 220 control subjects without chronic Q fever but with serological evidence of *C. burnetii* exposure and a risk factor for chronic Q fever were included in the study. Demographic and clinical characteristics of patients and controls are summarized in Table 2. Patients were slightly older than controls (2.4-year difference in median age). In both groups, vascular risk factors were more prevalent than cardiac valvular risk factors. Valvular risk factors, however, were more prevalent in the controls than in the patients. Patients with chronic Q fever were more likely than control subjects to be immunocompromised.

Association of SNPs in *MYD88*, *NOD2*, and *TLR1* With Chronic Q Fever

Genotyping of patients and controls was successful for all polymorphisms in genes encoding TLRs, NOD2, MyD88, TIRAP, $\alpha_v\beta_3$ integrin, and CR3 presented in Table 3. For each polymorphism, >94% of the subjects were genotyped. All SNPs were in Hardy–Weinberg equilibrium in the control group, except for *ITGB3* rs4642 ($\chi^2 = 6.18$); this SNP was excluded from further analysis.

In the analysis of all patients with chronic Q fever, a significantly different distribution of 1 *NOD2* and 1 *MYD88* polymorphism was revealed in the dominant model: *NOD2* L1007fsX1 ($P = .02$; OR, 3.34; 95% CI, 1.22–9.14) and *MYD88* –938C>A

Table 2 Baseline Patient Characteristics in Patients With Chronic Q Fever and Controls

Characteristic	Chronic Q Fever Cohort (n = 139)	Control Cohort (n = 220)	*P* Value[a]
Age median (IQR), y	70.0 (63.0–75.6)	67.6 (58.3–74.3)	.02
Male sex, No. (%)	114 (82)	169 (77)	.29
Classification of chronic Q fever, No. (%)			
Proven (%)	92 (66.2)	0	
Probable (%)	47 (33.8)	0	
Cardiovascular risk factor for chronic Q fever, No. (%)[b]			
Vascular aneurysm/prosthesis	95 (68.3)	129 (58.6)	.07
Valvular defect/prosthesis	39 (28.1)	111 (50.5)	<.001
Immunocompromised state[c]	21 (15.1)	14 (6.4)	.01

Abbreviation: IQR, interquartile range.

[a] *P* values based on Fisher exact test for categorical variables and Mann–Whitney *U* test for continuous variables.

[b] Nine case patients and 22 controls had both vascular and valvular risk conditions.

[c] In case patients, immunocompromised states included autoimmune disease with immunosuppressive drug treatment (n = 14), renal insufficiency (n = 1), renal transplantation (n = 1), cancer (n = 3), and prednis(ol)one use (n = 1); in controls, autoimmune disease with immunosuppressive drug treatment (n = 5), renal transplantation (n = 2), cancer (n = 4); and prednis(ol)on use (n = 4).

(*P* = .04; OR, 2.15; 95% CI, 1.05–4.39) (Table 4). Compared with controls, patients were more often heterozygous for the allelic variant of these SNPs.

When only patients with proven chronic Q fever were considered, *TLR1* R80T was distributed significantly different among patients and controls in the dominant model (*P* = .03; OR, 0.48; 95% CI, .24–.95) (Table 5), whereas it was marginally significant (*P* = .08; OR, 0.61; 95% CI, .35–1.06) when all patients (including those with probable Q fever) were included. Patients were less often heterozygous or homozygous for the allelic variant of this SNP. The distributions of *NOD2* L1007fsX1 and *MYD88* –938C>A showed a trend toward significance among patients and controls (*P* = .07 and *P* = .09, respectively) when only patients with proven chronic Q fever were included in the analysis. No associations were observed between polymorphisms in *TLR2*, *TLR4*, *TLR6*, *TIRAP*, *ITGAV*, *ITGB3*, or *ITGAM* and the development of chronic Q fever.

Table 3 Genotyped SNPs in genes encoding immune receptors and adaptor molecules

Gene	SNP ID	Gene region	Nucleotide change[a]	Amino Acid Change
TLR1	rs4833095	Exon 4	T > C	S248N
	rs5743611	Exon 4	G > C	R80T
TLR2	rs111200466	5′ UTR	ins > del	NA
	rs5743704	Exon 3	C > A	P631H
	rs5743708	Exon 3	G > A	R753Q
TLR4	rs4986790	Exon 3	A > G	D299G
	rs1927911	Intron 1	C > T	NA
TLR6	rs1039559	Intron 1	T > C	NA
	rs5743818	Exon 2	T > G	Synonymous (A644A)
TLR8	rs3747414	Exon 3	C > A	Synonymous (I751I)
	rs3764880	Exon 1	A > G	M1V affects protein start
NOD2	rs1077861	Intron 10	A > T	NA
	rs2066847	Exon 11	– > C	frameshift and stopcodon (L1007fsX1)
	rs2066844	Exon 4	C > T	R702W
MYD88	rs4988453	Promoter	C > A	NA
	rs6853	3′ UTR	A > G	NA
TIRAP	rs8177348	Intron 1	C > T	NA
	rs8177374	Exon 5	C > T	S180L
ITGAV	rs3738919	Intron 17	C > A	NA
	rs9333288	Intron 3	A > G	NA
ITGB3	rs3809865	3′ UTR	A > T	NA
	rs4642	Exon 10	G > A	Synonymous (E511E)
ITGAM	rs1143679	Exon 3	G > A	R77H
	rs1143678	Exon 30	C > T	P1146S

Abbreviations: SNP, single nucleotide polymorphism; ID, identification number; Ins, insertion; NA, not applicable; SNP, single-nucleoide polymorphism; UTR, untranslated region.
[a] The first nucleotide is the most common nucleotide.

Table 4 Associations of polymorphisms in immune receptors and adaptors genes and susceptibility to chronic Q fever

Polymorphism	Genotype distribution				Dominant model analysis		Recessive model analysis	
				*P*value[a]	*P*value[b]	OR (95% CI)[b]	*P*value[b]	OR (95% CI)[b]
***TLR1* rs4833095**	**TT**	**CT**	**CC**	.60	.40	1.21 (0.78-1.87)	.48	1.42 (0.53-3.77)
controls (%)	121 (57.6)	80 (38.1)	9 (4.29)					
patients (%)	71 (53.0)	55 (41.0)	8 (5.97)					
***TLR1* rs5743611**	**GG**	**GC**	**CC**	.21	.08	0.61 (0.35-1.06)	.77	0.78 (0.14-4.30)
controls (%)	158 (75.6)	47 (22.5)	4 (1.91)					
patients (%)	112 (83.6)	20 (14.9)	2 (1.49)					
***TLR2* rs111200466**	ins/ins	ins/del	del/del	.48	.67	0.90 (0.56-1.46)	.36	1.64 (0.56-4.79)
controls (%)	145 (69.4)	57 (27.3)	7 (3.35)					
patients (%)	93 (71.5)	30 (23.1)	7 (5.38)					
***TLR2* rs5743704**	**CC**	**CA**	**AA**	.69	.67	1.19 (0.54-2.59)	na	
controls (%)	193 (92.3)	16 (7.66)	0					
patients (%)	122 (91.0)	12 (8.96)	0					
***TLR2* rs5743708**	**GG**	**GA**	**AA**	.89	.51	1.34 (0.56-3.18)	.75	1.58 (0.10-25.46)
controls (%)	207 (94.5)	11 (5.02)	1 (0.46)					
patients (%)	129 (92.8)	9 (6.47)	1 (0.72)					
***TLR4* rs4986790**	**AA**	**AG**	**GG**	.19	.36	0.75 (0.41-1.38)	1.00	2.55E9 (0.0-∞)
controls (%)	174 (82.9)	36 (17.1)	0					
patients (%)	116 (86.6)	17 (12.7)	1 (0.75)					
***TLR4* rs1927911**	**CC**	**TC**	**TT**	.48	.25	1.30 (0.83-2.01)	.90	1.06 (0.46-2.43)
controls (%)	130 (61.9)	65 (31.0)	15 (7.14)					
patients (%)	74 (55.6)	49 (36.8)	10 (7.52)					
***TLR6* rs1039559**	**TT**	**TC**	**CC**	.81	.50	1.18 (0.72-1.94)	.90	1.03 (0.60-1.78)
controls (%)	60 (28.7)	108 (51.7)	41 (19.6)					
patients (%)	34 (25.4)	73 (54,5)	27 (20.1)					

***TLR6* rs5743818**	**TT**	**TG**	**GG**	.34	.19	0.75 (0.48-1.16)	.24	0.62 (0.27-1.38)
controls (%)	104 (49.5)	84 (40.0)	22 (10.5)					
patients (%)	76 (56.7)	49 (36.6)	9 (6.72)					
***TLR8* rs3747414 [c]**	**allele C**	**allele A**		.74				
controls (%)	276 (66.3)	140 (33.7)						
patients (%)	173 (65.0)	93 (35.0)						
***TLR8* rs3764880 [c]**	**allele A**	**allele G**		.15				
controls (%)	319 (77.1)	95 (22.9)						
patients (%)	193 (72.0)	75 (28.0)						
***NOD2* rs1077861**	**AA**	**TA**	**TT**	.89	.74	1.08 (0.70-1.67)	.64	1.18 (0.58-2.40)
controls (%)	97 (46.6)	91 (43.8)	20 (9.62)					
patients (%)	60 (44.8)	59 (44.0)	15 (11.2)					
***NOD2* rs2066847**	**-/-**	**-/C**	**C/C**	**.02**	**.02**	**3.34 (1.22-9.14)**	na	
controls (%)	204 (97.1)	6 (2.86)	0					
patients (%)	122 (91.0)	12 (8.96)	0					
***NOD2* rs2066844**	**CC**	**CT**	**TT**	.50	.48	1.27 (0.66-2.45)	na	
controls (%)	196 (89.5)	23 (10.5)	0					
patients (%)	121 (87.1)	18 (12.9)	0					
***MYD88* rs4988453**	**CC**	**CA**	**AA**	**.04**	**.04**	**2.15 (1.05-4.39)**	na	
controls (%)	195 (92.9)	15 (7.14)	0					
patients (%)	115 (85.8)	19 (14.2)	0					
***MYD88* rs6853**	**AA**	**GA**	**GG**	.52	.30	1.29 (0.80-2.07)	.78	0.78 (0.14-4.32)
controls (%)	155 (73.8)	51 (24.3)	4 (1.90)					
patients (%)	92 (68.7)	40 (29.9)	2 (1.49)					
***TIRAP* rs8177348**	**CC**	**CT**	**TT**	.23	.26	1.29 (0.83-2.01)	.12	1.93 (0.84-4.45)
controls (%)	128 (61.8)	68 (32.9)	11 (5.31)					
patients (%)	74 (55.6)	46 (34.6)	13 (9.77)					

Table 4 Continued

Polymorphism	Genotype distribution				Dominant model analysis		Recessive model analysis	
				*P*value[a]	*P*value[b]	OR (95% CI)[b]	*P*value[b]	OR (95% CI)[b]
TIRAP rs8177374	CC	CT	TT	.77	.76	0.93 (0.58-1.50)	.58	1.57 (0.31-7.91)
controls (%)	156 (71.9)	58 (26.7)	3 (1.38)					
patients (%)	102 (73.4)	34 (24.5)	3 (2.16)					
ITGAV rs3738919	CC	CA	AA	.70	.54	0.87 (0.56-1.36)	.69	1.14 (0.60-2.19)
controls (%)	82 (39.2)	102 (48.8)	25 (12.0)					
patients (%)	57 (42.5)	59 (44.0)	18 (13.4)					
ITGAV rs9333288	AA	AG	GG	.88	.70	1.09 (0.71-1.67)	.81	0.90 (0.37-2.20)
controls (%)	123 (56.4)	81 (37.2)	14 (6.42)					
patients (%)	75 (54.3)	55 (39.9)	8 (5.80)					
ITGB3 rs3809865	AA	TA	TT	.17	.07	0.67 (0.44-1.03)	.27	0.68 (0.34-1.35)
controls (%)	86 (39.4)	103 (47.2)	29 (13.3)					
patients (%)	68 (49.3)	57 (41.3)	13 (9.42)					
ITGAM rs1143679	GG	GA	AA	.68	.65	0.88 (0.49-1.56)	.57	1.60 (0.32-8.04)
controls (%)	180 (82.2)	36 (16.4)	3 (1.37)					
patients (%)	116 (84.1)	19 (13.8)	3 (2.17)					
ITGAM rs1143678	CC	CT	TT	.51	.49	1.19 (0.73-1.95)	.27	1.87 (0.62-5.70)
controls (%)	159 (75.7)	45 (21.4)	6 (2.86)					
patients (%)	97 (72.4)	30 (22.4)	7 (5.22)					

Abbreviations: na, not applicable.
[a] Fisher's exact test. [b] Logistic regression. [c] X-chromosomal.

Table 5 Distribution of *TLR1* (R80T), *NOD2* (L1007fsX1) and *MYD88* (-938C>A) genotypes in proven chronic Q fever patients (n=92) compared to controls (n=220)

Polymorphism	genotype distribution				Dominant model analysis		Recessive model analysis	
				P value[a]	*P* value[b]	OR (95% CI)[b]	*P* value[b]	OR (95% CI)[b]
***TLR1* rs5743611**	**GG**	**GC**	**CC**	.047	**.034**	**0.48 (0.24-0.95)**	.86	1.17 (0.21-6.48)
controls (%)	158 (75.6)	47 (22.5)	4 (1.91)					
patients (%)	78 (86.7)	10 (11.1)	2 (2.22)					
***NOD2* rs2066847**	**-/-**	**-/C**	**C/C**					
controls (%)	204 (97.1)	6 (2.86)	0	.067	.065	2.87 (0.94-8.79)	na	na
patients (%)	83 (92.2)	7 (7.78)	0					
***MYD88* rs4988453**	**CC**	**CA**	**AA**	.121	.091	2.00 (0.89-4.5)	na	na
controls (%)	195 (92.9)	15 (7.14)	0					
patients (%)	78 (86.7)	12 (13.3)	0					

Abbreviations: na, not applicable.
[a] Fisher's exact test.
[b] Logistic regression.

Effect of TLR1 SNP R80T on In Vitro IL-10 Production by *C. burnetii*–Stimulated Whole Blood

The functional consequences of *TLR1* R80T, *NOD2* L1007fsX1 and *MYD88* –938C>A were studied using *C. burnetii* stimulation of whole blood obtained from 93 control subjects with different *TLR1* R80T, *NOD2* L1007fsX1, and *MYD88* –938C>A genotypes.

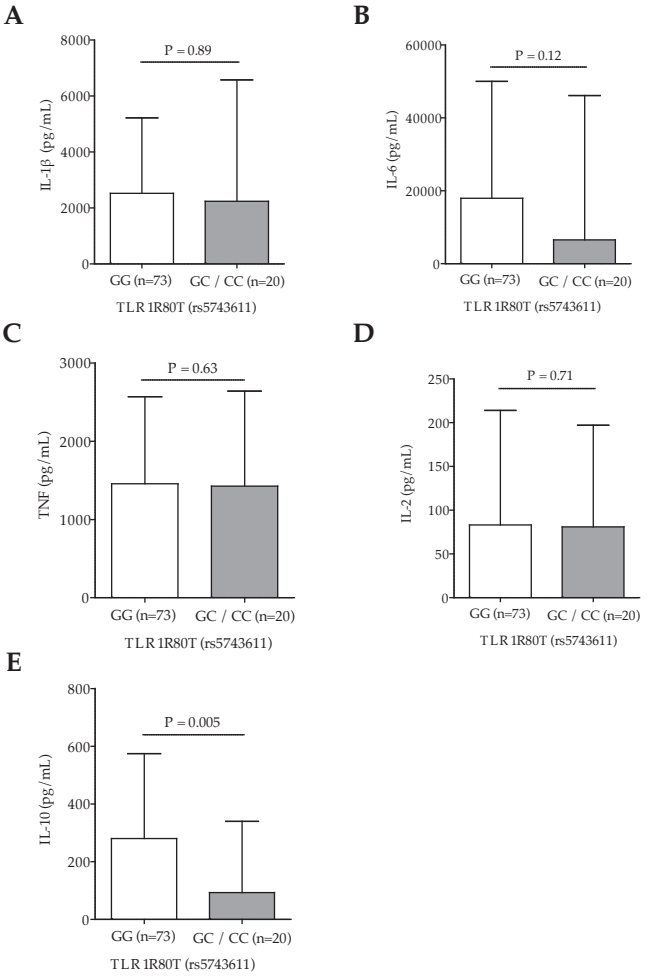

Figure 1. Association between *TLR1* R80T genotypes and cytokine production after whole-blood stimulation with *Coxiella burnetii*. IL-1β, interleukin 1β; IL-2, interleukin 2; IL-6, interleukin 6; IL-10, interleukin 10; TNF, tumor necrosis factor. Stimulation was performed for 24 hours with heat-killed *C. burnetii* Nine Mile phase I (RSA 493) (bacteria count, 1×10^7/mL). Data are presented as medians and interquartile ranges, and groups were compared with the Mann–Whitney *U* test.

Production of IL-1β, TNF, IL-2, IL-6, and IL-10 was measured after 24 hours of stimulation. Decreased IL-10 production was observed after stimulation with *C. burnetii* by blood cells with *TLR1* 80T containing genotypes, which is associated with lower susceptibility to chronic Q fever. Proinflammatory cytokine production, including IL-1β, IL-6, TNF, and IL-2, did not differ among *TLR1* R80T genotypes (Figure 1).

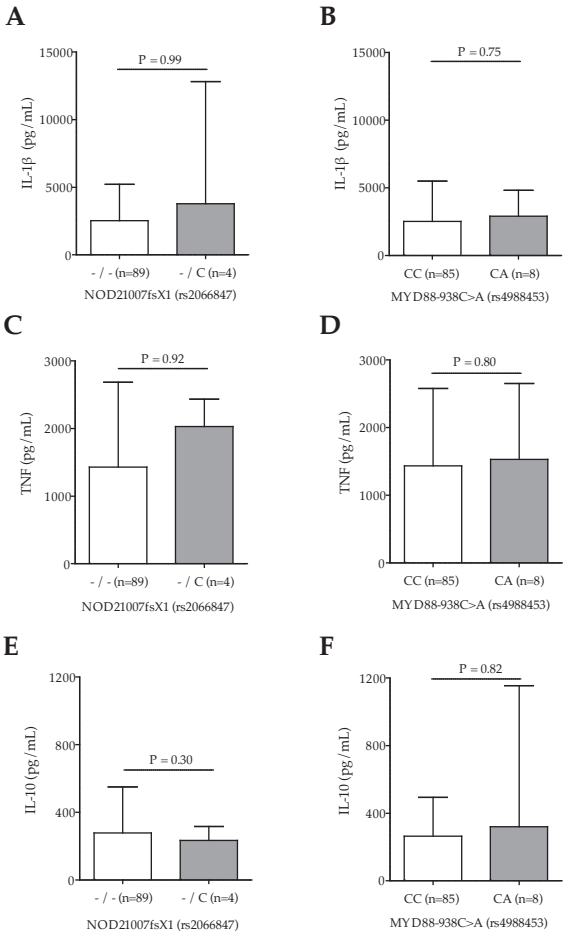

Figure 2. Association between *NOD2* and *MYD88* genotypes and cytokine production after whole-blood stimulation with *Coxiella burnetii*. Subjects were stratified by genotype for *NOD2* 1007fsX1 (*A*, *C*, and *E*) and *MYD88* –938C>A (*B*, *D*, and *F*). Stimulation was performed for 24 hours with heat-killed *C. burnetii* Nine Mile phase I (RSA 493) (bacteria count, 1×10^7/mL). Data are presented as medians and interquartile ranges, and groups were compared with the Mann–Whitney *U* test. Abbreviation: IL-1β, interleukin 1β; IL-10, interleukin 10; TNF, tumor necrosis factor.

There were no significant differences in the association between *NOD2* L1007fsX1 and *MYD88* –938C>A polymorphisms and the production of IL-1β, TNF, and IL-10 in response to *C. burnetii* (Figure 2). Findings for IL-6 and IL-2 are not shown (P = .78 and P = .58 respectively, with stratification by *NOD2* 1007fsX1 genotype; P = .51 and P = .52 with stratification by *MYD88* –938C>A genotype).

Discussion

In the present study, we investigated whether genetic variants in PRRs and adaptor proteins were associated with chronic Q fever in *C. burnetii*–infected individuals with vascular or valvular risk factors. Our results showed that *TLR1* R80T (rs5743611), *NOD2* L1007fsX1 (rs2066847) and *MYD88* –938C>A (rs4988453) are linked to the development of chronic Q fever. In addition, blood cells from individuals carrying the *TLR1* 80T variant that led to decreased susceptibility to chronic Q fever showed lower IL-10 production with *C. burnetii* stimulation. We observed no influence of *TLR2, TLR4, TLR6, TLR8, TIRAP, ITGAV, ITGB3,* and *ITGAM* SNPs on the susceptibility to chronic Q fever.

To our knowledge, this is the first genetic study investigating the role of SNPs in PRR and adaptor molecule genes in the development of chronic Q fever, the most severe complication of *C. burnetii* infection. Our study comprises the largest chronic Q fever cohort ever described. Previous studies on genetic predisposition to Q fever either concerned small cohorts of patients or focused on other clinical Q fever manifestations (acute Q fever or Q fever fatigue syndrome). Everett et al [27] studied susceptibility to acute Q fever by focusing on *TLR2* and *TLR4* SNPs, for which they demonstrated no correlation in 89 patients with acute Q fever and 162 controls. Helbig et al [28] investigated association between variants in *HLA*, *IFNG*, *IL10,* and *TNFR* genes and chronic Q fever or post–Q fever fatigue in 22 and 31 patients, respectively; they described significant differences in the frequencies of *IL10* promoter microsatellites in chronic Q fever compared with findings in 181 individuals without Q fever exposure. Vollmer-Conna et al [29] demonstrated that functional polymorphisms in *IFNG* and *IL10* affect disease severity and duration after serologically confirmed acute Q fever.

We focused on polymorphisms in immune genes involved in the initial recognition of *C. burnetii,* based on the assumption that chronic Q fever develops only after inefficient early recognition resulting in inadequate early clearance of the bacterium. Previous studies have shown that patients with chronic Q fever exhibit a different cytokine response to *C. burnetii* [30–32], but it is not clear whether this different response is specific for the myeloid cells of these individuals or due to the persistent presence of the bacteria in monocytes/macrophages. Deciphering

genetic polymorphisms in immune genes of the innate response to *C. burnetii* may give more insight into the mechanism.

In genetic association studies, the choice of the control group is of pivotal importance. We deliberately chose a control cohort of subjects living in the same Q fever–endemic area, with both past *C. burnetii* infection (as shown by positive anti–*C. burnetii* serological results) and cardiovascular risk factors for chronic Q fever, who did not develop chronic Q fever. When a different distribution of genetic polymorphisms is observed between patients and these controls, it indicates that these polymorphisms modify the risk of developing chronic Q fever, and not that of contracting *C. burnetii* infection or any underlying predisposing factors. The control cohort, however, was somewhat younger, had significantly more valvular risk factors, and was less immunocompromised than the patient cohort.

Notably, antimicrobial drug treatment at the time of acute Q fever might decrease the risk of chronic Q fever. Although information on previous treatment for acute Q fever was not obtained from the controls in this study, we assume that the majority of controls did not receive antibiotic treatment for acute Q fever because they participated in screening programs to detect previous *C. burnetii* infection.

The patient cohort included patients with proven or probable diagnosis of chronic Q fever; possible cases were left out. Given that the diagnosis of chronic Q fever is difficult—because direct detection of the bacterium is not possible in many cases—a probable chronic Q fever diagnosis is based on indirect evidence of persistent *C. burnetii* infection. These patients also receive long-term antibiotic treatment, with treatment and follow-up protocols identical to those used for proven chronic Q fever, but some of these patients may have no persistent infection. However, we also performed an analysis including only the subset of patients with proven chronic Q fever, which increased the level of diagnostic certainty for chronic Q fever in the case patients but decreased the power to detect associations.

We identified a significant association between the *TLR1* R80T polymorphism and the risk of chronic Q fever. This *TLR1* SNP has been described as a risk factor for invasive aspergillosis after hematopoietic stem cell transplantation [16], candidemia [33], and inflammatory bowel disease [34]. *TLR1* S248N, which is not linked to *TLR1* R80T, did not show an association with the development of chronic Q fever. We studied the functional consequences of the *TLR1* R80T polymorphism in a subset of controls. The presence of *TLR1* 80T allele resulted in significantly lower production of the anti-inflammatory cytokine IL-10 but did not affect TNF, IL-1β, or other proinflammatory cytokines, as reported elsewhere [5]. The *TLR1* 80T allele was significantly less frequent in the patients with proven Q fever than in the control cohort. This suggests that having lower IL-10 production on contact with *C. burnetii* may protect against chronic Q fever. This finding is in line with the

role of the anti-inflammatory cytokine IL-10 in the development of human chronic Q fever, which has been extensively reported [30, 35–37]. In short, IL-10 induces *C. burnetii* replication in naive monocytes [35]. In addition, IL-10 production in unstimulated peripheral blood mononuclear cells was increased after *C. burnetii* infection in individuals who subsequently developed chronic endocarditis relative to those who did not [36]. Moreover, transgenic mice that constitutively overexpress IL-10 is the only efficient model for chronic Q fever so far [38].

NOD2 is a cytoplasmic receptor involved in bacterial peptidoglycan sensing. The intracellular localization of NOD2 and the survival of *C. burnetii* in the intracellular PV may lead to ongoing interaction between them. The active secretion of bacterial proteins by *C. burnetii* through the PV membrane, demonstrated to occur by a type IV secretion apparatus [39], may play a role in this process. Genetic variation in *NOD2* has previously been linked to autoinflammatory diseases, such as Crohn disease and Blau syndrome [40]. An association with susceptibility to tuberculosis and leprosy has also been described [41, 42]. The *NOD2* L1007fsX1 SNP, leading to a frame shift and a premature stop codon, has a large effect on the protein. Its association with Crohn disease has been extensively described [43, 44]. Ammerdorffer et al [5] showed that *C. burnetii*– induced cytokine production by human mononuclear cells is mediated through NOD2, with NOD2-deficient individuals having strongly decreased IL-1β and IL-6 responses. In the present study, we were unable to show a significant effect on *C. burnetii*–induced cytokine responses when we stratified for the polymorphism *NOD2* L1007fsX1, most likely because of the rare occurrence of heterozygotes for the allelic variant and absence of homozygotes.

To our knowledge, the role of the adaptor molecule MyD88 in *C. burnetii* infection has not been studied previously. We found that *MYD88* −938C>A was significantly associated with susceptibility to chronic Q fever. In general, MyD88 plays a central role in innate immune responses, being downstream of all TLRs (except TLR3) and the interleukin 1 receptor. MyD88 deficiency leads to recurrent infections with pyogenic bacteria in early childhood but seems redundant for most other infections [15]. The *MYD88* −938C>A SNP has been shown to decrease promoter activity [45] and was found to be associated with development of sarcoidosis [46].

TLR2 is a receptor for bacterial lipopeptides, which are recognized by either TLR2/TLR1 or TLR2/TLR6 heterodimers. Although multiple studies have shown that TLR2 is involved in the host's immune response against *C. burnetii* [5, 47–49], we found no association between the TLR2 SNPs included in this study and development of chronic Q fever. This could indicate either that TLR2 has no prominent role in the elimination of *C. burnetii* or that the consequences of these genetic variants for the protein function in *C. burnetii* defense are limited. We also

did not observe any association of chronic Q fever with *TLR4, TLR6, TLR8,* or *TIRAP* polymorphisms. Genetic polymorphisms in the genes for the receptors $\alpha_v\beta_3$ integrin and CR3, which have been reported to be involved in *C. burnetii* uptake and TNF production [*7,* 50], were also not distributed significantly differently between patients with chronic Q fever and controls.

Our study has some limitations that need to be taken into account. Although this is the largest cohort of patients with chronic Q fever ever described, it is still relatively small for a study of the genetic associations of an infectious disease. We tried to overcome this limitation by using a control group almost similar in sex, age, and cardiovascular risk factors and with exposure to the same virulent *C. burnetii* strain. Another important consideration is that no correction for multiple testing was performed, because only candidate genes with a known or suspected role in *C. burnetii* recognition were investigated, and not randomly selected genes. It has to be taken into account that when correction for multiple testing is applied, the statistical significance of the *NOD2, MYD88,* and *TLR1* SNPs association with susceptibility to chronic Q fever is lost. To confirm our current findings, they need to be replicated in another cohort of patients with chronic Q fever.

In conclusion, our current findings suggest an association between *TLR1, NOD2,* and *MYD88* polymorphisms and the risk or developing chronic Q fever after infection with *C. burnetii*. Interestingly, we found that the protective *TLR1* 80T allele was associated with decreased *C. burnetii*–induced IL-10 production. Further research is warranted to elucidate the exact role of these receptors and the adaptor molecule in host defense against *C. burnetii* in humans, which could be used to strategize approaches to risk assessment, prophylactic treatment, or targeted therapy of chronic Q fever.

Notes

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4

Interferon-γ response against *C. burnetii* by peripheral blood mononuclear cells in chronic Q fever

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Submitted

Abstract

Background: Q fever is caused by *Coxiella burnetii,* an intracellular bacterium that infects mononuclear phagocytes. In some individuals, it causes a persistent cardiovascular infection: chronic Q fever. The aim of the present study was to investigate whether the *C. burnetii*-induced IFN-γ response was defective in chronic Q fever patients.

Methods: IFN-γ was measured in supernatants of *C. burnetii*-stimulated peripheral blood mononuclear cells (PBMCs) of chronic Q fever patients (n=17) and healthy individuals (n=17). To assess whether the IFN-γ-induced effects are intact, expression profiles of IFN-γ-induced genes of *C. burnetii*-stimulated PBMCs were compared between 6 patients and 4 healthy individuals. Neopterin, a marker of cellular immune response, was measured in PBMC culture supernatants and in sera of patients and healthy individuals. In a genetic association study, polymorphisms in genes involved in the Th1-cytokine response were analysed in patients (n=139) and at risk controls (n=220).

Results: IFN-γ production in vitro by *C. burnetii*-stimulated PBMCs of chronic Q fever patients, was significantly higher than in controls. In gene-expression analysis, genes downstream of IFN-γ were also strongly up-regulated in patients. Neopterin levels were significantly higher in supernatants of *C. burnetii*-stimulated PBMC of chronic Q fever patients than of controls. In addition, high serum levels of neopterin were found in patients. The *IL12B* polymorphisms rs3212227 and rs2853694 were associated with chronic Q fever. There was no association for polymorphisms in *IFNG*, *IFGR1*, *IL18* or *IL12RB1*.

Conclusion: IFN-γ production, as well as the response to IFN-γ, appears to be intact in chronic Q fever patients. Genetic analysis showed that polymorphisms in the IL-12p40 gene are associated with chronic Q fever.

Introduction

Q fever is a zoonosis caused by the Gram-negative coccobacillus *Coxiella burnetii*. The infection is transmitted to humans by inhalation of aerosols that contain bacteria derived from dried animal manure or birthing fluids [1]. During infection, more than 50% of the individuals remain asymptomatic, but in some acute Q fever develops [1, 2]. Regardless of initial manifestations, a minority of individuals develops a chronic infection that may become apparent months or years after initial exposure [3, 4]. Chronic Q fever mainly develops in people with pre-existing valvular disease, aortic aneurysm or vascular prostheses [5-7], and may present as endocarditis, a mycotic aneurysm, or vascular prosthesis infection, life-threatening conditions that need long-term antibiotics and sometimes surgical intervention [6, 8]. An immunosuppressed state as well as older age, pregnancy and renal insufficiency are also associated with increased risk for chronic Q fever [5, 9-11].

During the 2007-2011 Q fever outbreak in the Netherlands, the majority of patients with valvular/vascular risk factors for chronic Q fever apparently cleared the infection [12, 13]; however, more than 250 patients did develop chronic Q fever [14]. It is unknown what explains this difference in susceptibility to develop a chronic infection.

As an obligate intracellular pathogen, *C. burnetii* proliferates in mononuclear phagocytes, i.e., monocytes and tissue macrophages. *C. burnetii* uniquely replicates in an intracellular acidic vacuole that harbours late endosome markers, containing lysosomal enzymes and having a low pH [15-17]. In general, containment of intracellular infection by the host's immune system requires a pro-inflammatory response with granuloma formation and intracellular killing or control of the bacterium in activated monocytes/macrophages. The key cytokine in this process is interferon-γ (IFN-γ), a well-studied proinflammatory T helper (Th)-1 cytokine, which activates macrophages, and is able to eliminate or control intracellular pathogens [18]. IFN-γ production is induced by type-1 cytokines secreted by monocytes/macrophages or dendritic cells,, most notably interleukin (IL)-12 and IL-18 [19, 20].

The important role of IFN-γ in the defense against *C. burnetii* is supported by the high mortality observed in IFN-γ -/- mice infected with *C. burnetii* [21]. In-vitro studies have also shown that recombinant IFN-γ induces killing of *C. burnetii* by THP-1 monocytic cells, and inhibits growth of *C. burnetii* in mouse fibroblasts [22-24]. It has been reported that chronic Q fever is associated with a defective antigen-driven lymphocyte proliferation to *C. burnetii* antigens, with intact response to other antigens [25]. A substantial amount of *C. burnetii*-specific IFN-γ is produced by healthy individuals after vaccination with killed *C. burnetii* and after natural

infection [26]. Based on these observations, it has been assumed that chronic Q fever patients have an inadequate IFN-γ response to *C. burnetii* that in turn leads to persistent infection [21, 23, 27], but direct evidence for this hypothesis is lacking. The aim of present study is to investigate the IFN-γ response to *C. burnetii* in chronic Q fever patients.

Materials and Methods

Ethics statement

The study was approved by the Ethical Committee of Radboud university medical center, Nijmegen, the Netherlands. Subjects were enrolled after providing written informed consent (or waiver when deceased [n=5], as approved by the Ethical Committee). Institutional Review Boards of participating hospitals approved the inclusion of patients and controls in this study. The study has been performed in accordance with the Declaration of Helsinki.

Subjects

For the peripheral blood mononuclear cells (PBMC) stimulation experiments and serum measurements as described below, 17 proven chronic Q fever patients, who visited the outpatient clinic at the department of internal medicine of the participating hospitals, were included. Chronic Q fever was diagnosed based on the guidelines by the Dutch chronic Q fever consensus group [28]. Seventeen healthy individuals without known history of Q fever, were included as controls in these experiments. Serum of patients and controls was collected and stored in aliquots at -80°C until use.

For the genetic analysis study, all probable or proven chronic Q fever patients [28], who visited the outpatient clinic at the departments of internal medicine of the participating hospitals, were asked to participate. The recruitment took place as described before [29], and 139 patients were included. The control group consisted of 220 individuals from the same area with valvular or vascular abnormalities predisposing to chronic Q fever, who had serological evidence of exposition to *C. burnetii* (anti-*C. burnetii* phase II IgG antibodies ≥1:32) without clinical symptoms or serological evidence of chronic Q fever. These individuals were recruited as described previously [29].

Bacteria

C. burnetii Nine Mile (NM) phase I (RSA 493) – a reference strain isolated from a tick [30] – and *C. burnetii* 3262 – isolated from the placenta of a spontaneously aborted goat in the Netherlands [31] – were cultured in Buffalo Green Monkey cells

in a BSL-3 facility at the Central Veterinary Institute, as described previously [32]. The number of *Coxiella* DNA copies was determined using a Taqman real-time polymerase chain reaction [32]. The *C. burnetii* strains were inactivated by heating for 30 minutes at 99°C and stored at -80°C until use. Furthermore, Q-vax® vaccine (CSL Biotherapies, Victoria, Australia) [33] contains formaldehyde-inactivated *C. burnetii* Henzerling strain phase I in 50 μg/ml, and was used in a end-concentration of 100 ng/ml.

Isolation and stimulation of PBMCs

PBMCs from chronic Q fever patients and healthy individuals were isolated as previously described [34]. In short, venous blood was drawn from the cubital vein of all participants into 10 ml EDTA tubes. PBMCs were isolated from whole blood using density gradient centrifugation on Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden). The cells from the interphase were aspirated and washed twice in sterile PBS and resuspended in RPMI 1640 Dutch modification culture medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 1% L-glutamine, 1% pyruvate and 1% gentamicin. Cells were counted in a Coulter Counter Z® (Beckman Coulter, Fullerton, CA, USA), and adjusted to required concentration. The PBMCs [2.5 x 10^6/ml] were incubated in a round-bottom 96-wells plate (volume 200 μl/well) at 37°C and 5% CO_2 with either heat-killed *C. burnetii* NM [10^7/ml and 10^6/ml], heat-killed *C. burnetii* 3262 [10^6/ml], Q-vax [100 ng/ml] or heat-killed *C. albicans* (ATCC MYA-3573; UC820) [10^5 /ml] or culture medium alone. After 48 hours, supernatants were collected and stored at -20°C until being assayed.

IFN-γ and neopterin measurements

IFN-γ production was measured in the supernatants by enzyme-linked immunosorbent assay (ELISA; Pelikine compact, Sanquin, Amsterdam, the Netherlands), according to the manufacturer's instruction.

Neopterin was also measured in supernatants of eight patients and four healthy individuals by ELISA (IBL International, Hamburg, Germany), according to the manufacturer's instruction. Neopterin in serum was measured likewise, using stored sera of 21 chronic Q fever patients and 11 healthy controls.

Gene expression analysis

To assess gene expression, PBMCs of six patients and four healthy individuals were incubated for 8 hours in a flat-bottom 24-wells plate (volume 1 ml/well) at a concentration of [10^7 cells/ml] at 37°C and 5% CO_2 with heat-killed *C. burnetii* NM [10^7/ml], *E. coli* LPS [10 ng/ml] or culture medium alone.

RNA was extracted using the RNeasy Mini kit (Qiagen) and DNAse treatment. The quality of the RNA preparation was assessed using the 2100 Bioanalyzer and

the RNA 6000 Nano LabChip kit (Agilent Technologies), and its quantity was assessed using a Nanodrop. The PBMC gene expression was analyzed using Whole Human Genome 4×44K microarrays (Agilent Technologies, Massy, France), representing 45,000 probes and One-color Microarray Based Gene Expression Analysis kit, as previously described [35]. RNA samples of stimulated or unstimulated PBMCs from 6 chronic Q fever patients and 4 healthy individuals were included in the analysis.

The data were analyzed with R and the Bioconductor software suites. Raw data were preprocessed and quality-checked with *Agi4x44PreProcess* library and normalized through quantile normalization. Differential expression was assessed using *Limma* library. To explore IFN-γ pathway modulation in this dataset, nodes in the pathway were color-coded according to the ratio of gene expression in stimulated cells to gene expression in unstimulated cells.

Graphical representation of the pathway were drawn with Cytoscape [36]. Minimum Information About a Microarray Experiment (MIAME)-compliant data were submitted to the Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/) and can be assessed with the GEO series accession number GSE66476.

Genotyping of type-1 cytokine (receptor) polymorphisms and measuring IFN-γ response in whole blood according to genotype

From patients who came to the outpatient clinic, venous blood was drawn and stored at -80°C until use. DNA was isolated from these blood samples using standard methods [37]. Other participants, both patients and controls, received a buccal swab kit (Isohelix, Cell Projects Ltd., Harrietsham, Kent, UK) to obtain epithelial cells for DNA isolation. DNA was isolated using a buccal DNA isolation kit (Isohelix), according to the manufacturer's protocol. Single nucleotide polymorphisms (SNPs) were selected based on known functional effects on protein function or gene expression, published associations with human diseases and/or haploview data. In total, nine SNPs in *IFNG, IFNGR1, IL18, IL12B* and *IL12RB1* were genotyped with a Sequenom mass-spectrometry genotyping platform. Quality control was performed by duplicating 5% of the samples within and across plates, by the incorporation of positive and negative control samples and by sequencing samples to verify the various genotypes.

In a subgroup of the genotyped control subjects, whole blood assays were performed and *C. burnetii*-induced IFN-γ was measured, as described earlier [38]. In short, venous blood was drawn into 5 ml endotoxin-free lithium-heparin tubes (Vacutainer, BD Biosciences). Heat-killed *C. burnetii* NM RSA 493 phase I [10^7 bacteria/ml] was used as stimulus. After incubation, blood cultures were centrifuged and supernatants were stored at -20ºC until assayed.

Statistical analysis

Median IFN-γ and neopterin concentrations were compared using Mann-Whitney *U*-tests, using GraphPad Prism (GraphPad software Inc., version 5). Statistical tests were two-sided and a *P*-value below 0.05 was considered statistically significant. Presence of Hardy-Weinberg equilibrium (HWE) was analysed for all nine SNPs separately in the control cohort [39]. The difference in genotype frequencies between the patients and the control group were analyzed by means of a gene dosage model, with Fisher's exact test to determine significance. Subsequent dominant and recessive model analysis was performed by means of univariate logistic regression, for which odds ratios (ORs) and their 95% confidence intervals (95% CI) were reported. Because the choice of the genetic variants was based exclusively on genes products with an established role in response to *C. burnetii* recognition, rather than exploratory, no correction for multiple testing was performed. Statistical analyses were carried out with the IBM SPSS software (version 20).

Results

Intact *C. burnetii*–induced IFN-γ production by PBMCs of chronic Q fever patients

The ability of PBMCs of chronic Q fever patients to mount a recall response to *C. burnetii* was investigated by stimulation of PBMCs in the absence of serum for 48 hours with various *C. burnetii*-strains and subsequent measurement of IFN-γ in the supernatants. Figure 1 shows that a high IFN-γ production was induced in PBMCs of patients with all *C. burnetii*-strains and that this was significantly different from the response in PBMCs of healthy individuals. Interestingly, inactivated *C. albicans*, known to induce IFN-γ in PBMCs of healthy individuals, led to significant less IFN-γ production in chronic Q fever patients.

Transcriptome analysis reveals *C. burnetii*-induced upregulation of genes downstream of IFN-γ in chronic Q fever patients

Since chronic Q fever patients produce high amounts of IFN-γ upon *C. burnetii* stimulation, we wondered whether the pathway downstream of IFN-γ would be defective in chronic Q fever patients. In a whole-transcriptome microarray approach, the transcriptional responses of PBMCs to *C. burnetii* and, for comparison, to *E. coli* LPS were investigated.

Performing principal component analysis, the gene expression profiles overall showed a different activation pattern in patients and controls, and for patients also different between *C. burnetii* and *E. coli* LPS stimulation (not shown). For the

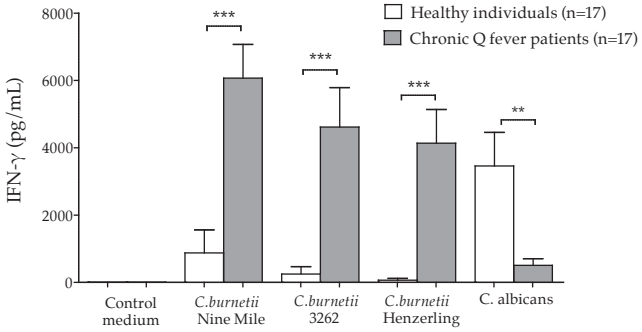

Figure 1. *C. burnetii*-induced IFN-γ production differs between chronic Q fever patients and healthy individuals. IFN-γ was measured in supernatant of PBMCs stimulated for 48 hours with inactivated *C. burnetii* Nine Mile [10^6/ml], *C. burnetii* 3262 [10^6/ml], *C. burnetii* Henzerling [100 ng/ml] (all phase I) and *Candida albicans* [10^5/ml]. Values are expressed as mean ± standard error. *P* values are calculated using Mann-Whitney U-test.
***P*<0.01, ****P*<0.001.

purpose of this study, we focused on the gene expression related to the IFN-γ downstream pathway. In Figure 2, the median gene modulation in inflammatory responses of PBMCs of six patients and four healthy controls is shown, in which the IFN-γ downstream pathway can be evaluated. As can be seen from this figure, PBMCs from patients show up-regulation of genes downstream of IFN-γ signaling when stimulated with *C. burnetii*, while this was not the case in healthy individuals. Stimulation with *E. coli* LPS, however, led to up-regulation of IFN-γ pathway in healthy individuals, which was less pronounced in chronic Q fever patients (Supplementary figure 1).

Neopterin levels are increased in chronic Q fever patients

Neopterin is a highly stable low molecular weight molecule that belongs to the chemical group known as pteridines. Its biosynthesis can be seen as a general marker of activation of the cellular immune system, in particular by IFN-γ action. In the course of a cellular immune reaction, neopterin can be measured in serum. It is produced *in vitro* by macrophages after stimulation with IFN-γ. We found significantly higher concentrations of neopterin in serum of chronic Q fever patients than in controls (Figure 3a). In addition, in the supernatant of *C. burnetii* NM-stimulated PBMCs of chronic Q fever patients, neopterin levels were significantly higher than of healthy individuals (Figure 3b), while in supernatant of *C. albicans*-stimulated PBMCs, neopterin concentrations were similar.

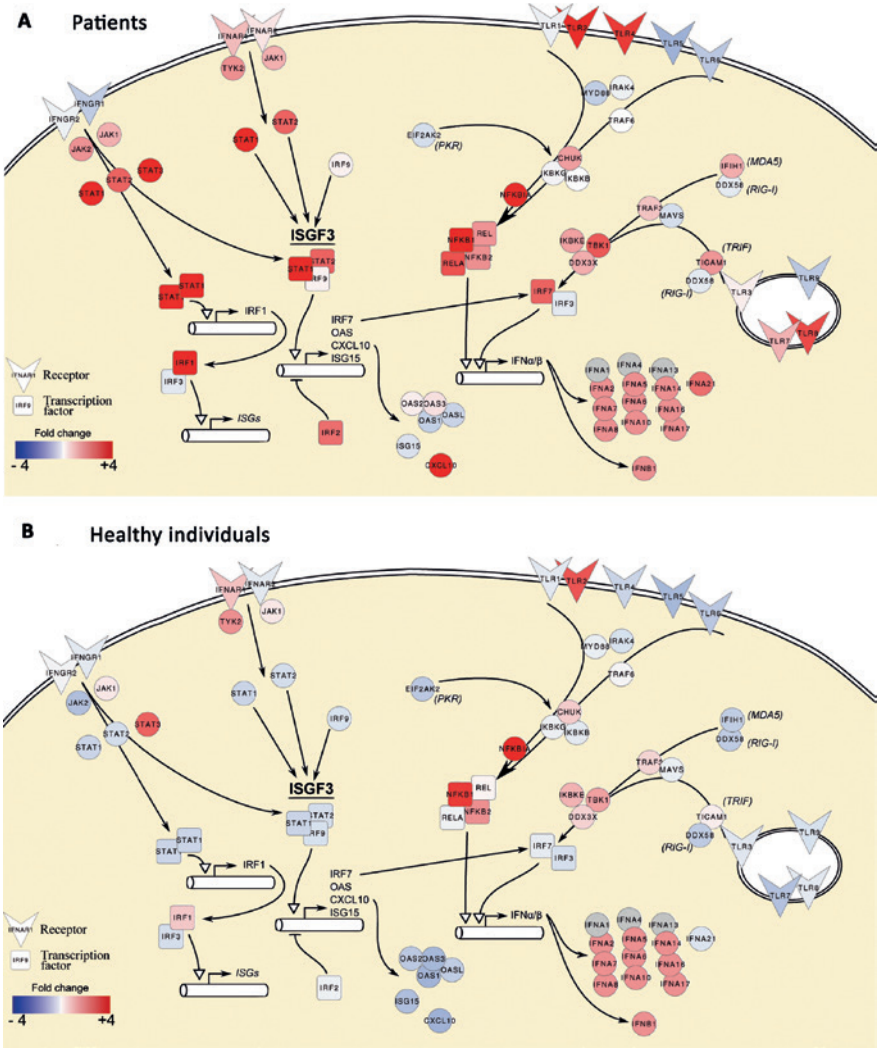

Figure 2. Differential expression of inflammatory response pathways, including the IFN-γ pathway, in *C. burnetii* stimulated PBMCs. PBMCs of six chronic Q fever patients (A) four healthy individuals (B) were stimulated with heat-inactivated *C. burnetii* Nine Mile [10^7/ml] for 8 hours. The median ratio of gene expression in stimulated PBMCs to the unstimulated PBMCs are color-coded from blue (Fold change [FC]= -4) to red (FC= +4) at each receptor or transcription factor.

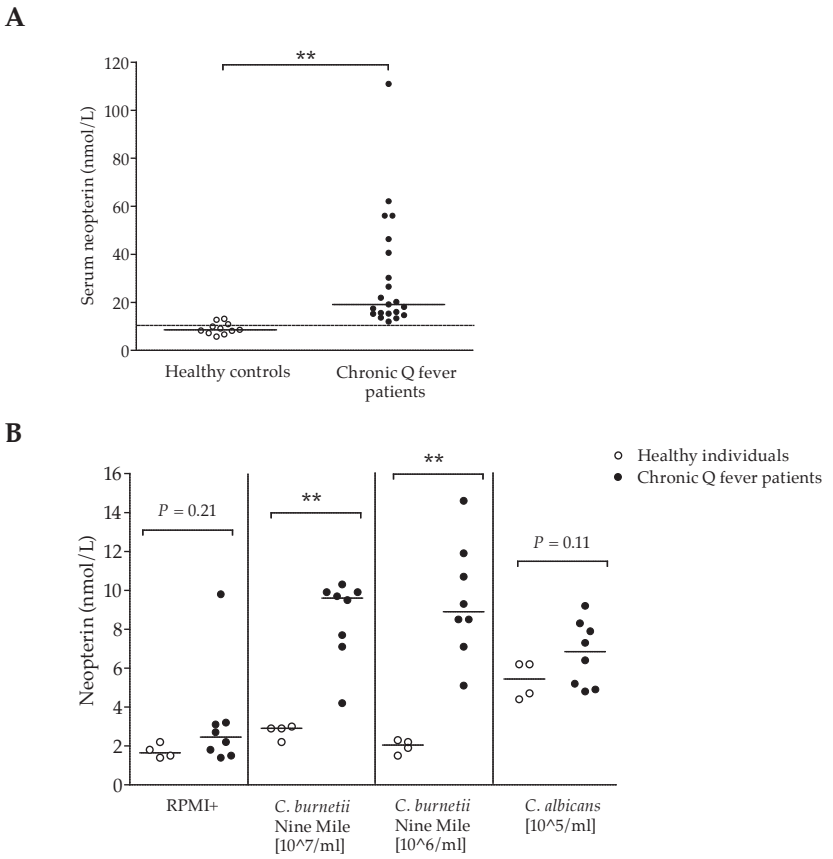

Figure 3. *C. burnetii*-induced neopterin is significantly different between chronic Q fever patients and healthy individuals. (A) Neopterin was measured in serum of healthy controls (n=11) and chronic Q fever patients (n=21). (B) Neopterin was measured in supernatant of PBMCs of patients (n=8) and of healthy individuals (n=4) stimulated for 48 hours with inactivated *C. burnetii* Nine Mile (NM) phase I [10^7/ml], *C. burnetii* NM phase I [10^6/ml] and *C. albicans* [10^5/ml]. Medians of healthy individuals and patients are compared by Mann-Whitney *U*-test. ** $P<0.01$.

Genetic polymorphisms in *IL12B* are differently distributed between chronic Q fever patients and at risk controls and are associated with decreased IFN-γ production

We investigated whether subtle, common genetic variations in the type-1 cytokine pathway, that affect IFN-γ production or response, are associated with the risk for development of chronic Q fever. We performed a genetic association study using a cohort of chronic Q fever patients and appropriate control subjects as described

previously [29]. In total, 139 (92 proven and 47 probable) chronic Q fever patients and 220 control subjects without chronic Q fever but with serological evidence of *C. burnetii* exposure and a risk factor for chronic Q fever were included. Genotyping of patients and controls was successful for all polymorphisms in genes encoding IFN-γ (*IFNG*), IFN-γ receptor chain 1 (*IFNGR1*), IL-18 (*IL18*), IL-12p40 (*IL12B*) and the IL-12 receptor β1 chain (*IL12RB1*) presented in Table 1. For each polymorphism, >92% of the subjects were genotyped. All SNPs were in Hardy-Weinberg equilibrium in the control group.

Table 1 Genotyped SNPs in genes encoding IFN-γ *(IFNG)*, IFN-γ receptor chain 1 *(IFNGR1)*, interleukin-18 *(IL18)*, interleukin-12p40 *(IL12B)*, and interleukin-12 receptor chain β1 *(IL12RB1)*

Gene	SNP ID	Gene region	Nucleotide change[a]	Amino Acid Change
IFNG	rs2069718	Intron 3	C > T	
	rs1861494	Intron 3	T > C	
IFNGR1	rs1327474	Promoter	A > G	
	rs2234711	5′ UTR	T > C	
IL18	rs549908	Exon 4	T > G	Synonymous (S35S)
IL12B	rs2853694	Intron 4	A > C	
	rs3212227	3′ UTR	A > C	
IL12RB1	rs436857	Exon 1	G > A	P40L
	rs11575934	Exon 7	A > G	R214Q

Abbreviations: SNP, single nucleotide polymorphism; ID, identification number.
[a] The first nucleotide is the most common nucleotide.

In the gene dosage analysis, genotyping revealed an association between chronic Q fever and both *IL12B* polymorphisms: *IL12B* rs2853694 (Fisher's exact *P*=0.006) and *IL12B* rs3212227 (*P*=0.004). No associations were observed between polymorphisms in *IFNG*, *IFNGR1*, *IL18*, *IL12RB1* and the presence of chronic Q fever (Table 2). Subsequently, *IL12B* rs2853694 was found to be significantly differently distributed in a recessive model analysis, with two C alleles leading to increased risk of chronic Q fever (*P* = 0.001; OR, 2.18 [95% CI, 1.35-3.53]). *IL12B* rs3212227 distribution was significantly different in a dominant model analysis, with protective effect of the C allele (*P* = 0.004; OR, 0.50 [95% CI, 0.31-0.80]) (Table 2). These two polymorphisms are not strongly linked (r^2=0.248).

Table 2 Associations of polymorphisms in *IFNG, IFNGR1, IL18, IL12B, IL12RB1* genes and development of chronic Q fever

Polymorphism	Genotype distribution				Dominant model analysis		Recessive model analysis	
				P value[a]	*P* value[b]	OR (95% CI)[b]	*P* value[b]	OR (95% CI)[b]
***IFNG* rs2069718**	CC	CT	TT	.36	.25	1.30 (0.83-2.05)	.25	1.51 (0.75-3.05)
controls (%)	86 (43.0)	96 (48.0)	18 (9.00)					
patients (%)	48 (36.6)	66 (50.4)	17 (13.0)					
***IFNG* rs1861494**	TT	CT	CC	.29	.58	1.13 (0.74-1.73)	.11	1.96 (0.85-4.50)
controls (%)	125 (57.3)	82 (37.6)	11 (5.05)					
patients (%)	75 (54.4)	50 (36.2)	13 (9.42)					
***IFNGR1* rs1327474**	AA	AG	GG	.67	.51	1.17 (0.74-1.86)	.40	1.24 (0.75-2.03)
controls (%)	70 (32.1)	100 (45.9)	48 (22.0)					
patients (%)	40 (28.8)	63 (45.3)	36 (25.9)					
***IFNGR1* rs2234711**	TT	CT	CC	.56	.37	0.82 (0.53-1.27)	.39	0.76 (0.41-1.42)
controls (%)	81 (37.0)	104 (47.5)	34 (15.5)					
patients (%)	58 (41.7)	64 (46.0)	17 (12.2)					
***IL18* rs549908**	TT	GT	GG	.14	.21	0.75 (0.49-1.17)	.28	1.50 (0.72-3.16)
controls (%)	89 (43.0)	102 (49.3)	16 (7.73)					
patients (%)	67 (50.0)	52 (38.8)	15 (11.2)					
***IL12B* rs2853694**	AA	AC	CC	**.006**	.26	1.32 (0.81-2.16)	**.001**	**2.18 (1.35-3.53)**
controls (%)	65 (31.3)	98 (47.1)	45 (21.6)					
patients (%)	34 (25.6)	49 (36.8)	50 (37.6)					
***IL12B* rs3212227**	AA	AC	CC	**.004**	**.004**	**0.50 (0.31-0.80)**	.07	0.15 (0.02-1.18)
controls (%)	122 (58.4)	77 (36.8)	10 (4.78)					
patients (%)	99 (73.4)	34 (25.4)	1 (0.75)					
***IL12RB1* rs436857**	GG	AG	AA	.72	.74	0.93 (0.59-1.46)	.56	1.31 (0.53-3.24)
controls (%)	133 (63.6)	65 (31.1)	11 (5.26)					
patients (%)	87 (65.4)	37 (27.8)	9 (6.77)					
***IL12RB1* rs11575934**	AA	AG	GG	1.00	.95	0.99 (0.64-1.51)	.94	0.97 (0.52-1.83)
controls (%)	97 (44.3)	93 (42.5)	29 (13.2)					
patients (%)	62 (44.6)	59 (42.5)	18 (13.0)					

[a] Fisher's exact test. [b] Logistic regression.

Next we investigated the functional consequences of these two genetic variations by assessing the *C. burnetii* induced IFN-γ production in whole blood samples from control subjects stratified for *IL12B* rs2853694 and rs3212227 genotypes. IFN-γ production was measured in supernatants after 24 hours of culture. These control subjects were stratified for genotype either in a recessive model (rs2853694; low-risk AA/AC versus high-risk CC) or in a dominant model (rs3212227; high-risk AA versus low-risk AC/CC) (Figure 4). The mean IFN-γ production by *C. burnetii*-stimulated blood cells in subjects with rs2853694 CC genotype was 243 pg/mL and in subjects with AA/AC genotype 563 pg/mL. a difference that was not significant (P=0.13). Subjects with rs3212227 AC/CC low-risk genotypes and CC genotypes neither showed statistically different mean IFN-γ production (573 pg/mL versus 428 pg/mL: P=0.40).

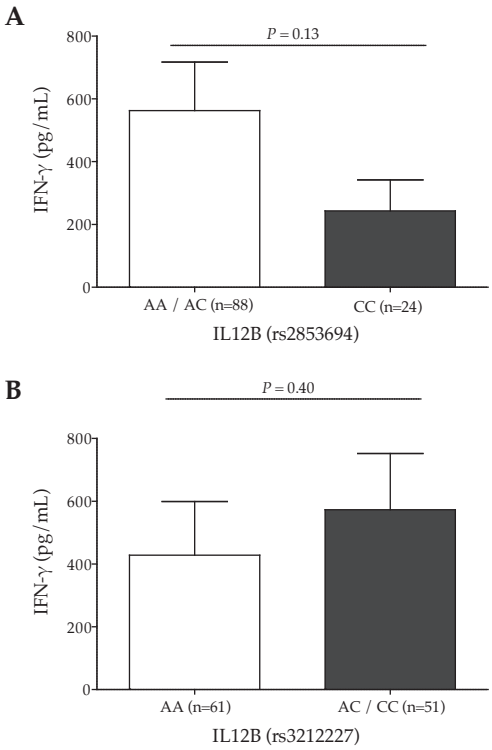

Figure 4. *IL12B* rs2853694 and rs3212227 genotypes and *C. burnetii*-induced IFN-γ in whole blood. In vitro stimulation of whole blood was performed for 24 hours with heat-inactivated *C. burnetii* Nine Mile phase I [1x 10^7 bacteria/mL]. Subjects were stratified by genotype (A) *IL12B* rs2853694 AA/AC versus CC, (B) *IL12B* rs3212227 AA versus AC/CC. Data are presented as means ± standard error of the mean. Groups were compared by Mann-Whitney *U*-test.

Discussion

In this study we investigated the *C. burnetii*-induced IFN-γ production in chronic Q fever patients. We show that immune cells of chronic Q fever patients are not only capable of a high IFN-γ production in response to *C. burnetii*, but also display up-regulation of genes downstream of the IFN-γ receptor in addition to production of neopterin, which is highly IFN-γ-dependent. We found that genetic polymorphisms in *IL12B*mare associated with the development of chronic Q fever.

Previously, it has been assumed that chronic Q fever patients have an inadequate T-cell derived IFN-γ production in response to *C. burnetii* infection. Our study could not confirm this assumption, and our findings provide evidence that *C. burnetii* induced IFN-γ production in chronic Q fever is rather increased. In a previous study, Koster et al [25] found that lymphocytes of chronic Q fever patients with endocarditis fail to proliferate *in vitro* in response to *C. burnetii* antigens. In one of these patients, this was demonstrated five years after the endocarditis was treated with antibiotics and cardiac valve replacement. This unresponsiveness was antigen-specific, since lymphocyte proliferation in response to *Candida* antigens was preserved [25]. The method of studying *C. burnetii*-specific adaptive immune responses used by Koster et al., i.e., lymphocyte proliferation, is different from our recent studies in which we showed increased IFN-γ production by ex-vivo *C. burnetii*-stimulated whole blood of chronic Q fever patients [40, 41]. In our present study, we confirmed these findings in PBMCs of chronic Q fever patients cultured for 48 hours in the presence of either one of three different *C. burnetii* strains. The absence of autologous serum in these cultures show that the IFN-γ production observed is not dependent on anti-*C. burnetii* antibodies.

Surprisingly, we found decreased IFN-γ response to *Candida albicans* in chronic Q fever patients compared to healthy individuals. This seems to indicate that PBMCs of patients with an active chronic Q fever infection are strongly responsive to *C. burnetii* in a specific fashion, at the expense of responsiveness to other stimuli. More research is necessary to unravel this mechanism.

The transcriptome analysis revealed that genes downstream of the IFN-γ receptor are upregulated in PBMCs of chronic Q fever patients upon stimulation with *C. burnetii*. In addition, IFN-dependent transcription factors were specifically upregulated in *C. burnetii* stimulated PBMCs of patients, and not in healthy individuals (data not shown). This shows that the IFN-γ signaling pathway is intact in chronic Q fever. The high neopterin levels in vivo – the measurements in serum – and in vitro – in *C. burnetii*-stimulated PBMC cultures – indicate that macrophages of chronic Q fever patients are activated by IFN-γ. This confirms that the IFN-γ signaling pathway is intact. In a previous study, mean neopterin levels in plasma of 13 acute and 23 chronic Q fever patients were increased (5.4 and 5.1

ng/mL, respectively) compared to 17 healthy individuals (2.1 ng/mL), although these differences were not significant [42].

Interestingly, our findings that the IFN-γ pathway in response to *C. burnetii* is intact in chronic Q fever patients can be added to the observation that anti-*C. burnetii* antibody titers are high in chronic Q fever patients [43] . Taken together, we may conclude that we have not found evidence for an impaired adaptive immune response in these patients. Since they are not able to kill the pathogen, apparently the antibody response and the strong IFN-γ response do not lead to adequate bactericidal effects. Why these hosts fail to eliminate the bacterium is still enigmatic.

It has been shown that IL-23 is the critical first signal from the antigen-presenting cells after recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). IL-23 signals through binding to the IL-23 receptor on T-cells, and stimulates cellular proliferation and early production of IFN-γ and TNF. IFN-γ in turn binds to the IFN-γ receptor on macrophages and activates, in synergy with TNF, various microbicidal pathways as well as production of IL-12. Subsequently, IL-12 drives, in synergy with IL-18, activation, differentiation and proliferation of T-cells along to the Th1-cells to produce more IFN-γ [44]. An essential component of both IL-12 and IL-23 is IL-12p40. In the present study, we found that the presence of SNPs in the promoter region (rs2853694) and at the 3'UTR (rs3212227) of the IL-12p40 gene, *IL12B*, were associated with the development of chronic Q fever. For both SNPs, association with leprosy, another disease caused by an intracellular pathogen, has been described [45, 46]. We found that subjects with the risk genotype of the polymorphisms did not significantly differ with regard to IFN-γ production. Hence we were unable to prove that these polymorphisms have an impact on the susceptibility to *C. burnetii* through production of IFN-γ. The high IFN-γ production upon *C. burnetii* stimulation that is seen in chronic Q fever patients is measured during an active chronic infection and most likely results from an ongoing stimulation of adaptive immune responses. In this light it would be interesting to compare initial IFN-γ responses between acute Q fever patients who eventually develop a chronic infection and those who do not. Honstettre et al. previously measured IL-12p40 release by PBMCs of patients with acute Q fever. They describe a trend of reduced IL-12p40 release during acute Q fever in patients with valvulopathy, of which 50% subsequently developed chronic Q fever [47].

There are, however, some considerations that should be taken into account when interpreting our results. First of all, we studied the immune response of circulation blood mononuclear cells to *C. burnetii*. It may be more relevant to study the local immune response in *C. burnetii* infected vascular walls or valvular tissue. Both vascular and valvular chronic *C. burnetii* infection are mostly low-grade

infections in which systemic immune activation is apparently not effective in clearing the local infection. The local immunological processes are likely crucial for survival of *C. burnetii* at predilection sites such as defective cardiac valves and aneurysmatic vascular wall. Immunohistochemical studies of *C. burnetii*-infected cardiac valves showed small, focal collections of infected mononuclear phagocytes [48, 49]. Further identification of these cells and their immunological environment could help us to better understand the local persistence of *C. burnetii*.

Second, the model that we used to study the IFN-γ response to *C. burnetii*, i.e., in-vitro stimulation of PBMCs with heat-inactivated bacteria, might not reflect the immunological processes in vivo. In particularly, relatively high doses of *C. burnetii* were used to stimulate the PBMCs in culture, compared to the low antigen load in serum during Q fever infection.

In conclusion, the present study shows that IFN-γ production and the response to IFN-γ appear to be intact in chronic Q fever patients and does not explain the failure to clear the infection. Although our genetic analysis showing that polymorphisms in the IL-12p40 gene are associated with chronic Q fever also points to the IFN-γ pathway, the functional consequences of these polymorphisms in Q fever are unclear.

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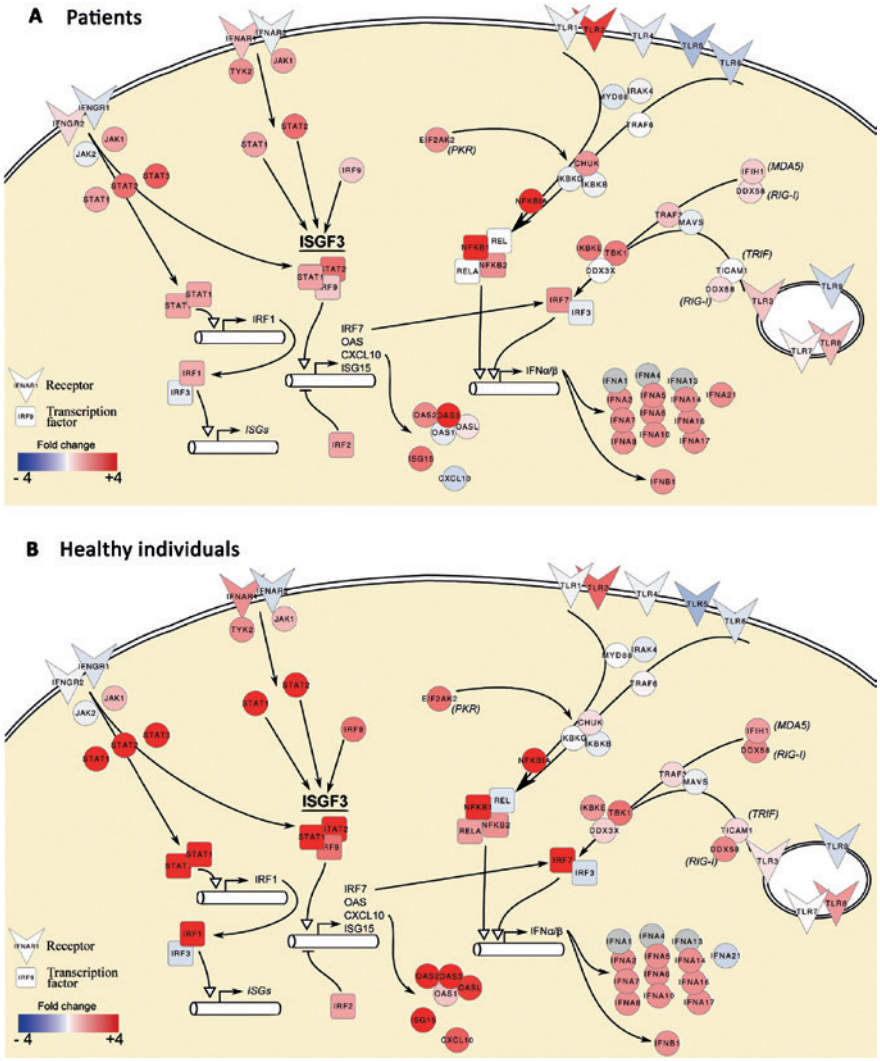
Conflict of interests

All authors declare that they have no conflict of interest.



Supplementary Figure 1. Differential expression of inflammatory response pathways, including IFN-γ pathway, in *E. coli* LPS stimulated PBMCs. PBMCs of six chronic Q fever patients (A) and four healthy individuals (B) were stimulated with *E. coli* LPS [10 ng/ml] for 8 hours. The median ratio of gene expression in stimulated PBMCs to the unstimulated PBMCs are color-coded from blue (Fold change [FC]= -4) to red (FC= +4) at each receptor or transcription factor.

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II

Detection of *C. burnetii* infection using cell-mediated immune responses

5

Specific Interferon-γ detection for the diagnosis of previous Q fever

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Abstract

Background. Current practice for diagnosis of Q fever, caused by the intracellular pathogen *Coxiella burnetii*, relies mainly on serology and, in prevaccination assessment, on skin tests (STs), which both have drawbacks. In this study, *C. burnetii*–specific interferon γ (IFN-γ) production was used as a new diagnostic tool for previous Q fever, circumventing most of these drawbacks. Our aim was to compare this test to serology and ST.

Methods. One thousand five hundred twenty-five individuals from an endemic area with a risk for chronic Q fever were enrolled. IFN-γ production was measured after in vitro stimulation of whole blood with *C. burnetii* antigens. Various formats using different *C. burnetii* antigens were tested. Serology and ST were performed in all individuals.

Results. In all assay formats, *C. burnetii*–specific IFN-γ production was higher ($P < .0001$) in seropositive or ST-positive subjects than in seronegative and ST-negative subjects. Whole blood incubated for 24 hours with *C. burnetii* Nine Mile showed optimal performance. After excluding subjects with equivocal serology and/or borderline ST results, IFN-γ production was 449 ± 82 pg/mL in the positive individuals (n = 219) but only 21 ± 3 pg/mL in negative subjects (n = 908). Using Bayesian analysis, sensitivity and specificity (87.0% and 90.2%, respectively) were similar to the combination of serology and ST (83.0% and 95.6%, respectively). Agreement with the combination of serology and ST was moderate (84% concordance; $\kappa = 0.542$).

Conclusions. Specific IFN-γ detection is a novel diagnostic assay for previous *C. burnetii* infection and shows similar performance and practical advantages over serology and ST. Future studies to investigate the clinical value in practice are warranted.

Editorial Commentary by Graves on pages 1752-53.

Introduction

Acute Q fever, caused by *Coxiella burnetii,* is often not recognized because it may have an asymptomatic or a mild course [1]. Chronic life-threatening endovascular infection may, however, develop, particularly in patients with valvulopathy or aneurysmas [1, 2]. A correct diagnosis of *C. burnetii* infection is crucial for patient care, as well as for vaccination and epidemiological studies.

The standard method to detect Q fever is measurement of specific antibodies [3, 4]. However, assessment of T-lymphocyte immunity might be an additional and even superior method because defense against this intracellular pathogen mainly depends on cellular immunity, including interferon γ (IFN-γ)–mediated macrophage activation [5–7]. Hence, a skin test (ST) can be done to assess delayed-type hypersensitivity to intradermally injected *C. burnetii*. The ST is almost exclusively used for prevaccination screening. Its disadvantages are variability, lack of a well-defined cutoff, and dependence on trained personnel to perform a ST. Therefore, we developed an in vitro IFN-γ production assay, in which IFN-γ production in blood after exposure to *C. burnetii* antigens is measured, similar to the quantiferon test for tuberculosis [8, 9].

Between 2007 and 2010, the Netherlands experienced the largest outbreak of Q fever worldwide [10], with an estimated >40 000 infected individuals and >250 patients with chronic Q fever [11, 12]. In 2010, the health authorities decided to offer vaccination with the *C. burnetii* whole-cell vaccine Q-vax [13] to inhabitants of the endemic area at risk for chronic Q fever (ie, those with preexisting heart valve lesion, prosthetic valve, congenital heart anomalies, aortic aneurysm, or vascular graft). Vaccine candidates were screened by serology and ST to exclude preexisting sensitization to *C. burnetii* antigens that may cause hypersensitivity reactions upon vaccination [14]. This prevaccination screening presented a unique opportunity to explore the applicability of our novel assay. We tested different formats of the assay and assessed its accuracy in diagnosing previous Q fever.

Methods

Study Population and Sampling Procedure

The study population consisted of vaccine candidates in the Dutch Q fever vaccination campaign from 21 January 2011 to 20 April 2011; the campaign coincided with a 30-fold decrease of new human Q fever cases in the spring compared with the peak in 2009, probably related to veterinary measures, including culling of all pregnant goats on infected farms. The target population consisted of individuals living in the highly endemic area based on surveillance data available. Voluntariness

and application by general practitioners were required. One week before the planned vaccination, candidates were invited to the Municipal Health Service in 's-Hertogenbosch. Blood was collected for serology followed by the ST. All candidates were asked to participate in the study by donating 5 mL of heparinized blood for the IFN-γ assay. This sample was obtained simultaneously with the serological sample and processed within 12 hours, as previous experiments had shown that samples assessed at 2 or 12 hours did not affect the assay results. IFN-γ production was not relevant for the decision whether or not to vaccinate. The study was performed according to the guidelines of the local ethics committee, and written informed consent was obtained. Demographic details were collected anonymously.

C. burnetii IFN-γ Production Assay

IFN-γ production was measured after in vitro stimulation of whole blood incubated under 2 different conditions (A or B) described below. Also, 2 *C. burnetii* antigens were used separately for stimulation. In parallel, stimulation with phytohemagglutinin was performed as a positive control and incubation with no reagent as a negative control.

In format A, undiluted whole blood was incubated in a closed tube at 37°C for 24 hours. In format B, the blood was diluted 1:5 in medium (Roswell Park Memorial Institute 1640 Dutch modification supplemented with glutamax [2 mM], pyruvate [1 mM], and gentamicin [1 mg/mL]) and incubated in 24-wells plates at 37°C and 5% carbon dioxide for 48 hours.

The 2 *C. burnetii* antigens were the Q-vax vaccine [15] and the heat-inactivated laboratory strain *C. burnetii* Nine Mile RSA 493 (*C. burnetii*–NM) [16], kindly provided by Dr D. Frangoulidis (Bundeswehr Institute of Microbiology, Munich, Germany).

Q-vax vaccine contains formaldehyde-inactivated *C. burnetii* Henzerling strain phase 1 in 50 μg/mL. Previous dose–response experiments in Q fever patients showed optimal IFN-γ production in the range of 100–500 ng/mL. A single lot (No. 0980– 07 201) was used and diluted to 100 ng/mL.

C. burnetii–NM phase 1 was cultured in a biosafety level 3 facility at the Central Veterinary Institute, using Buffalo Green Monkey cells in Earle's Modified Eagle Medium supplemented with 10% fetal calf serum, 1% nonessential amino acids, and 1% L-glutamine. The concentration of *C. burnetii* DNA was determined by real-time polymerase chain reaction. The supernatant was centrifuged for 15 minutes at 1000 × *g* and stored in aliquots at −80°C. Killing was done by heating for 30 minutes at 99°C. For stimulation *C. burnetii*–NM was used in an end concentration of 10^7 bacteria/mL, previously shown to be optimal in Q fever patients. The same batch was used for all assays.

After incubation of blood samples with either *C. burnetii* antigens, phytohemagglutinin (PHA), or nil, cultures were centrifuged and supernatants were stored at –20°C.

IFN-γ was measured by enzyme-linked immunosorbent assay. Serological and ST results were unknown to those performing the assay. Net IFN-γ production was expressed as the concentration of IFN-γ in stimulated samples minus that in negative controls. If either IFN-γ production in the negative control exceeded 24 pg/mL (thrice the lower detection limit of the ELISA) or the IFN-γ production after PHA stimulation was <24 pg/mL without the *C. burnetii*–stimulated aliquots exceeding 24 pg/mL, the assay was considered inconclusive.

Serology and Skin Testing

Antibodies against *C. burnetii* were determined in serum by indirect immunofluorescence measuring immunoglobulin M and immunoglobulin G against *C. burnetii*–NM phase 1 and 2. Seropositivity was defined as anti–*C. burnetii* titers ≥1:32. A solitary weak reactivity of immunoglobulin G against phase 2 antigens below the cutoff level of 1:32 was defined as equivocal because this was considered possible false positive or crossreacting. Seropositivity led to exclusion from vaccination.

Q-vax skin tests [15] were performed by professionals. In short, the local response to 0.1 mL intradermally injected Q-vax ST (containing the same *C. burnetii* antigens as Q-vax vaccine, but in a lower concentration) in the forearm was measured after 7 days. Only participants with negative or equivocal serological results were invited for ST reading. The ST results were classified as positive (induration ≥5 mm), borderline (induration 1–4 mm or any swelling and/or redness), or negative (no induration, swelling, or redness). Positive and borderline results led to exclusion from vaccination.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 and SAS version 9.2. Mann–Whitney *U* tests were performed to test statistical significance of IFN-γ production differences between groups. Association between inconclusiveness of specific IFN-γ production and demographics was assessed with a generalized linear model with identity link function [17]. Receiver operating characteristics (ROC) curves were created to assess accuracy (area under the curve [AUC]) of the different IFN-γ assay formats. Spearman's *r* was calculated to report correlation. Subsequently, a Bayesian model [18], in which no test is considered to be the gold standard, was used to determine the sensitivity and specificity of the IFN-γ assay of the best performing format. Using this method [18], adapted for several cutoffs, the ROC curve was estimated. The proportionate agreement and the

Cohen's κ were calculated to assess agreement [19]. The McNemar test was used to compare the proportion of positive results between tests. The level of significance was set at $P \leq .05$.

Results

During the prevaccination visit, 1786 individuals were screened. No subject had signs of acute Q fever (no immunoglobulin M level compatible with acute Q fever), whereas 12 had a serological profile compatible with chronic Q fever and were referred for further analysis [20]. Blood samples from 1525 individuals were obtained for IFN-γ production. Assay format A was performed on all 1525 samples; format B was discontinued after 1306 samples. Table 1 shows the characteristics of the vaccine candidates.

Table 1 Characteristics of the Total Population Participating in the Q Fever Prevaccination Screening and the Study Population

	All individuals in pre-vaccination screening (n=1786)	Study population (n=1525)
Mean age[a] ± SD (years)	63.5 ± 14.7	64.0 ± 14.2
Female sex (%)	668 (37.4)	558 (36.6)
Exclusion of vaccination based on:		
positive serology (%)	181 (10.1)	158 (10.4)
positive/borderline ST (%)	210 (11.8)	190 (12.5)
Serology indicating chronic Q fever infection	12 (0.7)	9 (0.6)

Abbreviations: ST, skin test.
[a] Age at 01-01-2011.

Results of the IFN-γ measurements in format A and B were conclusive for 1278 (83.8%) and 1223 (93.6%) individuals, respectively. After excluding individuals with equivocal serology or borderline ST results, 1127 (73.9%) and 1087 (83.2%) individuals were left with a conclusive outcome for all 3 tests (Figure 1).

Assay format A showed inconclusive results in 247 (16.2%) individuals; format B had only 83 (6.4%) inconclusive results. Inconclusiveness in format A was associated with age and sex ($P = .02$ and $P = .0008$ respectively; no interaction, $P = .40$), being most frequent in female subjects aged >80 years (29%; 95% confidence interval [CI],

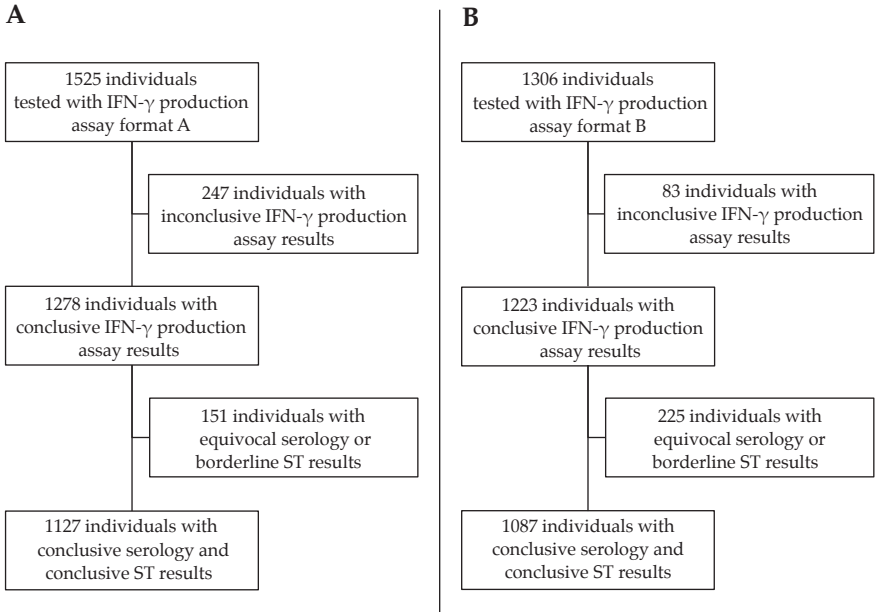

Figure 1. Flow diagrams of individuals included in the specific interferon γ (IFN-γ) production measurement. The numbers of individuals are shown separately for the IFN-γ assay in format A and format B. Format A: IFN-γ production assay in undiluted whole blood incubated 24 hours; format B: IFN-γ production assay in 1:5 diluted blood incubated 48 hours. Abbreviations: IFN-γ, interferon γ; ST, skin test

21–38) and least frequent in male subjects aged <80 years (13%; 95% CI, 11–15). Only 41 of the 247 inconclusive results in format A were due to high background IFN-γ production in the control sample; these were judged as technical errors or low-grade inflammation. Two hundred six results were inconclusive because of absent PHA response in the absence of a clear antigen-specific response (judged as anergy). However, 157 of 206 (76%) had an adequate PHA response in the corresponding sample in format B.

Subjects with conclusive results for all 3 tests were divided, based on the combined outcome of serology and ST, in seropositive or ST-positive (previous *C. burnetii* exposure) or seronegative and ST-negative (no *C. burnetii* exposure) groups. In the 1127 subjects with conclusive results in format A, 218 were seropositive or ST-positive, and 908 were seronegative and ST-negative. In the 1087 subjects with conclusive results in format B, 190 were seropositive or ST-positive, and 897 were seronegative and ST-negative. The IFN-γ production (mean ± standard error) was compared between these groups in both formats separately (Figure 2*A* and 2*B*).

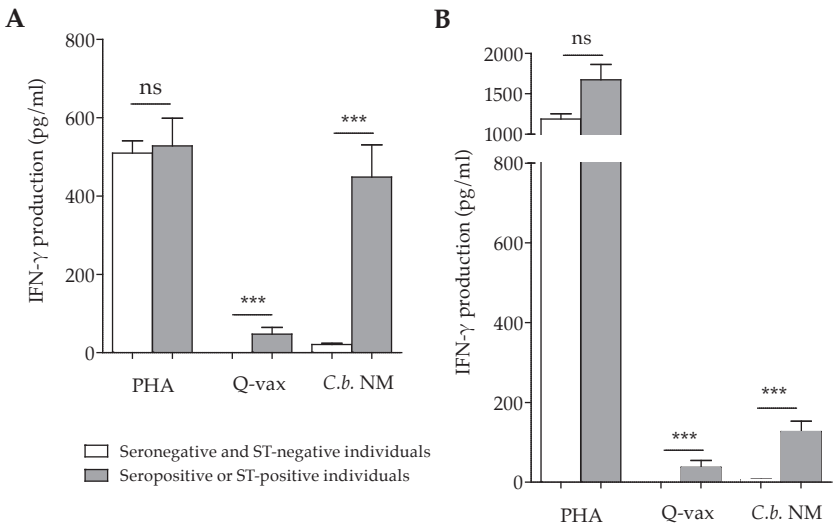

Figure 2. *Coxiella burnetii*–specific interferon γ (IFN-γ) production (pg/ mL) in seronegative and skin test (ST)–negative individuals (white bars) compared with seropositive or ST-positive individuals (gray bars). *A*, IFN-γ production (undiluted whole blood incubated 24 hours) in 908 seronegative and ST-negative individuals compared with 219 seropositive or ST-positive individuals. *B*, IFN-γ production (1:5 diluted blood incubated 48 hours) in 897 seronegative and ST-negative individuals compared with 190 seropositive or ST-positive individuals. Values are expressed as mean ± standard error. *P* values are calculated using Mann–Whitney *U* test. ***$P < .0001$. Abbreviations: *C.B.*-NM, *Coxiella burnetii* Nine Mile; IFN-γ, interferon γ; NS, not significant; PHA, phytohemagglutinin; ST, skin test.

PHA-induced IFN-γ production did not distinguish between individuals with previous and no *C burnetii* exposure. However, *C. burnetii* antigen–specific IFN-γ production was significantly higher in subjects with previous exposure. IFN-γ production in assay format A stimulated with *C. burnetii*–NM distinguished best between seropositive or ST-positive individuals and negative individuals; IFN-γ production was 449 ± 82 pg/mL in the sero- or ST-positive individuals and 21 ± 3 pg/mL in the seronegative or ST-negative individuals, respectively.

The ROC curves (Figure 3), combining serology and ST as (surrogate) gold standard, revealed the highest AUC for format A with *C. burnetii*–NM (AUC, 0.8452). The correlation between *C. burnetii*–NM– and Q-vax–induced IFN-γ production in format A and between the *C. burnetii*–NM– induced IFN-γ production in format A and format B are shown in a scatter plot (Figure 4). The Spearman's rank correlations were 0.5410 and 0.637, respectively ($P < .0001$).

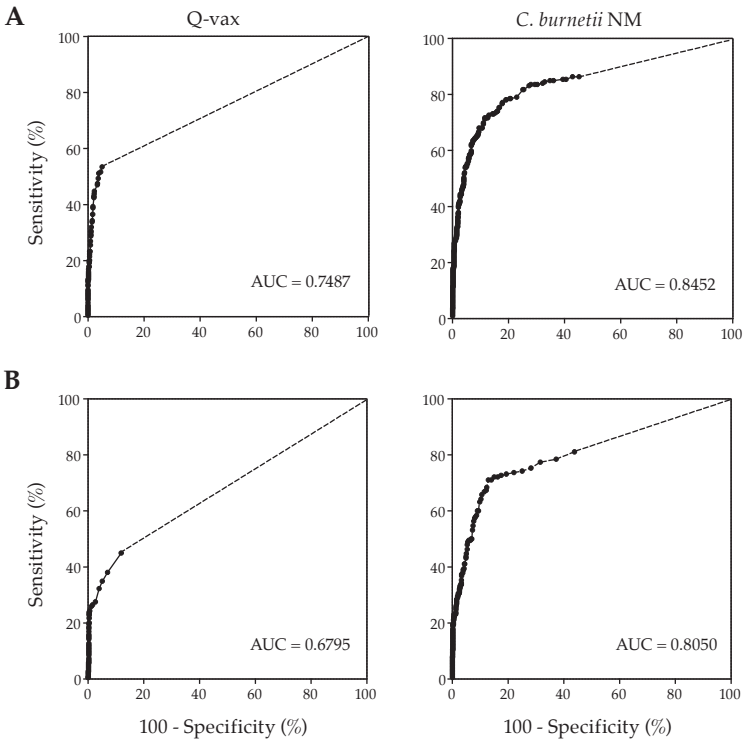

Figure 3. Comparison of the receiver operating characteristic (ROC) curves of the interferon γ (IFN-γ) production assay in different formats considering positive serology or skin test (ST) outcome as case and negative serology and ST outcome as control. ROC curves of the IFN-γ production assay in 2 different formats (*A* and *B*) and with two *Coxiella burnetii* antigens are shown. *A*, IFN-γ production in undiluted whole blood incubated 24 hours. *B*, IFN-γ production in 1:5 diluted blood incubated 48 hours. The area under the curve indicates the accuracy of the IFN-γ production assay. Abbreviations: AUC, area under the curve; NM, Nine Mile.

Comparison With Serology and Skin Testing

We focused on format A using *C. burnetii*–NM for further analysis. Figure 5 shows the data of 1278 individuals with conclusive results in format A, stratified according to the outcome of serology and ST, including equivocal serology and borderline ST results (see also Supplementary Table 1, showing the other IFN-γ assay formats). Specific IFN-γ (mean ± standard error) production was calculated in each group. This was highest in positive serology outcome, followed by positive ST outcome in combination with equivocal or negative serology. IFN-γ production was lowest in seronegative and ST-negative subjects. In borderline ST outcome, IFN-γ production

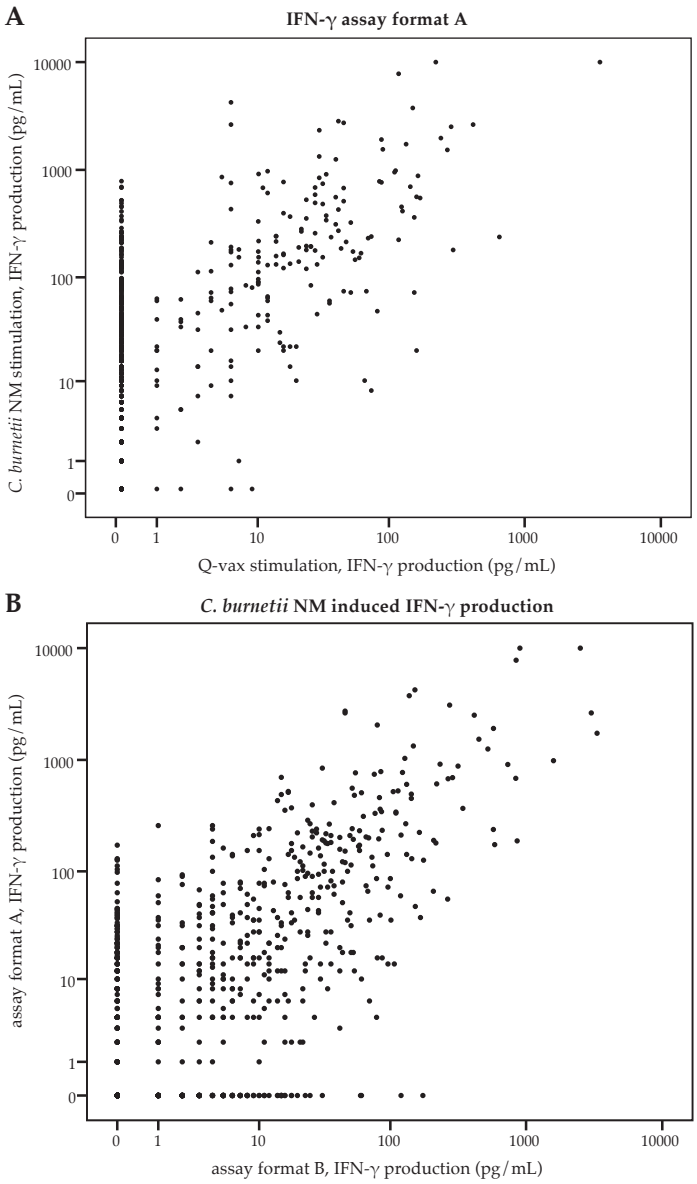

Figure 4. Scatter plots showing the correlation in interferon γ (IFN-γ) production between Q-vax and *Coxiella burnetii*–Nine Mile (NM) stimulation within IFN-γ assay format A (upper plot) and the correlation in IFN-γ production induced by *C. burnetii*–NM between assay format B and A (lower plot). The Spearman's rank correlations were 0.5410 and 0.637, respectively ($P < .0001$). Every dot represents 1 subject, sofar as dots are not overlapping. Abbreviations: IFN-γ, interferon γ; NM, Nine Mile.

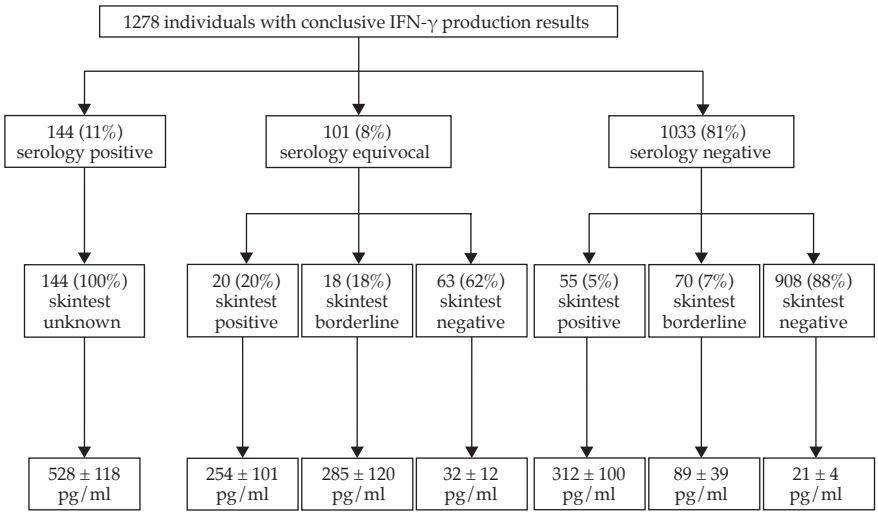

Figure 5. Distribution of serology and skin test outcome and interferon γ (IFN-γ) production in the study population with conclusive IFN-γ production results in assay format A (n = 1278). The upper boxes indicate the number (%) of individuals. The lowest boxes indicate the specific IFN-γ production (mean ± standard error; pg/mL) in whole blood incubated for 24 hours and stimulated with *Coxiella burnetii* Nine Mile. Abbreviation: IFN-γ, interferon γ.

was not significantly different between those with equivocal and negative serological findings ($P = .07$).

Using Bayesians statistics, we determined sensitivity and specificity of the IFN-γ assay at predefined cutoff values from 16 to 80 pg/mL, equivalent to 2–10 times the lower detection limit for the enzyme-linked immunosorbent assay. For the 1127 individuals with conclusive results for the IFN-γ assay, serology and ST were analyzed. A noninformative prior distribution was used for all parameters. The prior prevalence of *C. burnetii* infection was given a flat prior distribution over the range 0–0.5.

The prior sensitivity and specificity of the tests were given a flat prior distribution over the range 0–1. The data of the performance of the IFN-γ measurement at different cutoffs are shown in Table 2. The resulting ROC curve for the IFN-γ assay (Figure 6) shows an accuracy (AUC) of 0.9239 (95% credible interval [CrI], 0.8466–0.9756). Choosing an optimal cutoff value of 32 pg/mL, sensitivity and specificity of the IFN-γ assay were 87.0% and 90.2% (95% CrI, 72.2–98.8 and 86.0–94.5, respectively). The sensitivities and specificities of combined serological and ST, as calculated in this analysis, were 83.0% and 95.6% (95% CrI, 70.1–98.7 and 93.3–99.4, respectively). The prevalence of past Q fever was estimated in this

Table 2 Sensitivity and Specificity With Concomitant 95% Credible Interval of the Specific Interferon γ Production at Cutoff Values in the Range of 16–80 pg/mL

Cutoff value for specific IFN-γ production (pg/ml)	Sensitivity (%)	95% CrI (%)	Specificity (%)	95% CrI (%)
16	91.8	78.4-99.7	82.4	78.1-86.7
24	87.9	73.7-98.6	87.1	82.8-91.5
32	87.0	72.2-98.8	90.2	86.0-94.5
40	82.3	67.8-94.9	92.5	88.4-96.7
48	79.2	65.0-92.4	94.0	90.4-98.0
56	78.3	63.9-91.4	94.9	91.3-98.7
64	76.3	62.1-89.3	95.9	92.4-99.4
72	73.0	58.9-85.4	96.1	92.7-99.5
80	70.3	56.3-83.1	96.9	93.6-99.8

A Bayesian model was used with flat priors for prevalence and test parameters, including IFN-γ production and the combination of serology and skin test. The IFN-γ production measurement was performed in 24 hrs incubated whole blood, stimulated with *C. burnetii* Nine Mile. Only individuals with conclusive results for all three tests were included (n=1127).
Abbrevations: 95% CrI, 95% credible interval.

analysis to be 19.3% (95% CrI, 14.9–25.0). In comparison, available data on blood donors in the high-incidence area in 2009, based on serological screening alone, show a *C. burnetii* immunoglobulin G prevalence of 12.2% [21].

Agreement between the dichotomized IFN-γ assay (with cutoff of 32 pg/mL) and the combination of the other 2 tests is shown in Table 3. Overall agreement was 84% (949 of 1127) with $\kappa = 0.542$ ($P < .0001$), which is considered moderate [22]. The proportion of individuals with past or present *C. burnetii* infection identified by the IFN-γ assay, but not by serology or ST, was significantly higher (118 of 178 = 0.66) than the proportion identified by serology and ST but not by the IFN-γ assay (60 of 178 = 0.34) (McNemar $\chi^2 = 18.90$; $P < .0001$).

Performance in Equivocal Serology

We examined more closely the data in the 101 individuals with equivocal serology to know if the ST and the IFN-γ production assay (at cutoff level of 32 pg/mL) data revealed agreement in identifying individuals with and without previous Q fever (Figure 7). In 16 of 20 individuals with positive ST, IFN-γ production was above the

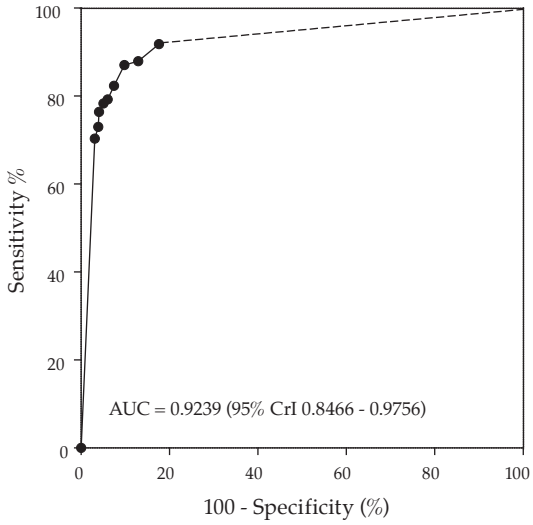

Figure 6. Receiver operating characteristic curve of the best performing interferon γ (IFN-γ) production assay at cutoff values ranging from 16 to 80 pg/mL using a Bayesian model without taking serology and skin test (ST) as gold standard. The IFN-γ production measurement was performed in whole blood incubated 24 hours and stimulated with *Coxiella burnetii* Nine Mile. Only individuals with conclusive results for all 3 tests were included (n = 1127). The area under the curve with concomitant 95% credible interval indicates the accuracy of the IFN-γ production measurement. Abbreviations: AUC, area under the curve; CrI, credible interval.

Table 3 Relationship Between the Interferon γ Production Outcome and the Combination of Serology and Skin Test Outcome

	Combination of serology and ST outcome[b]		Total
	positive	negative	
IFN-γ production measurement outcome[a]			
Positive	159	118	277
Negative	60	790	850
Total	219	908	1127

The IFN-γ production measurement was performed in 24 hrs incubated whole blood, stimulated with *C. burnetii* Nine Mile. Only individuals with conclusive results for all three tests were included (n=1127). Overall agreement was 84% (949/1127), κ=0.542 ($P < 0.0001$). Abbreviations: ST, skin test.

[a] IFN-γ production measurement was considered positive at value > 32 pg/mL and considered negative at value ≤ 32 pg/mL.

[b] Combination of serology and ST was considered positive if either one was positive and considered negative if both were negative.

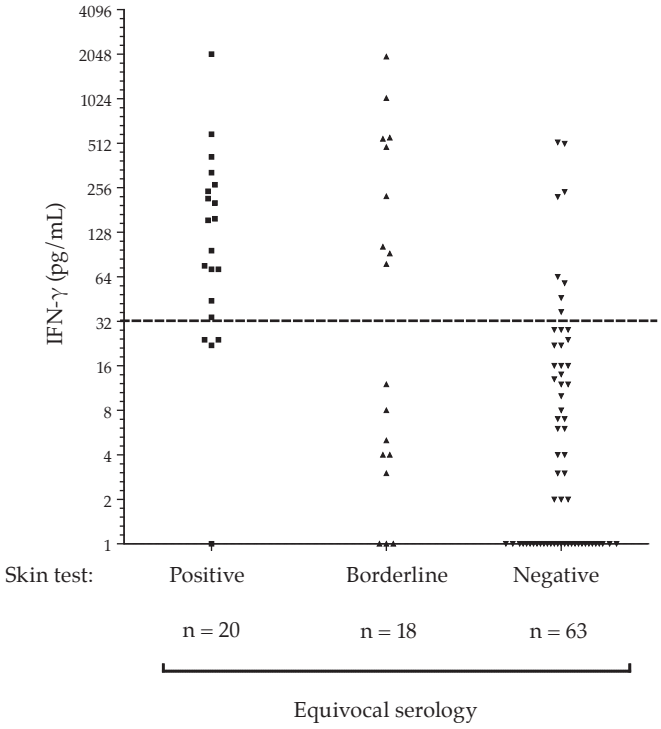

Figure 7. Interferon γ (IFN-γ) production (pg/mL) according to skin test results in individuals with equivocal serology. The individuals with conclusive results in the IFN-γ production assay in format A stimulated with *Coxiella burnetii* Nine Mile with equivocal serology are included (n = 101). The dotted line indicates the cutoff of 32 pg/mL. Abbreviation: IFN-γ, interferon γ.

cutoff, whereas in 55 of 63 individuals with negative ST the IFN-γ production was below the cutoff. Excluding those individuals with borderline ST, agreement between IFN-γ assay and ST was 71 of 83 (85.6%) in cases with equivocal serology. In borderline ST results (n = 18), the IFN-γ results were clearly separated in high and low IFN-γ clusters. Thus, the IFN-γ assay has additional value in identifying (past) Q fever in individuals with equivocal serology and borderline ST results.

Discussion

In a sample of >1500 individuals at risk for chronic Q fever, we demonstrated that an assay that measures antigen-specific IFN-γ production in vitro is a valuable tool in the diagnosis of Q fever. The accuracy of this test is similar to the traditional serology and ST. The test is easy to perform: no advanced personnel or equipment are required for incubation, and read-out and the data are available the next day. It is patient friendly by excluding the need for in vivo testing, which, in sensitized individuals, can be troublesome.

The IFN-γ production assay was analyzed in individuals with a specific risk for development of chronic Q fever and considerable *C. burnetii* exposure. The assay was carried out in 2 formats with different incubation periods and 2 different *C. burnetii* antigens. The results of the 4 assay conditions were consistent, showing higher IFN-γ production in individuals with positive serology or ST response, whereas PHA stimulation was not specific. Thus, *C. burnetii*–specific IFN-γ production seems to be a useful diagnostic aid in determining previous Q fever infection.

The IFN-γ production in undiluted blood exposed to *C. burnetii*–NM for 24 hours showed the highest AUC, using serology and ST as the putative gold standard. This format performed better than in 1:5 diluted blood. This may be because of higher concentrations of serum factors, the 5× higher inoculum, and the shorter incubation in the undiluted assay. Stimulation with *C. burnetii*–NM performed better than Q-vax. In the ROC curves, Q-vax stimulation reached maximal sensitivity of 53%. The scatter plot data support this finding by showing no Q-vax–induced IFN-γ in many samples with *C. burnetii*–NM– induced IFN-γ production. The sensitivity of the assay may be strain dependent; the Henzerling strain of Q-vax may be less potent than *C. burnetii*–NM in inducing IFN-γ in the Dutch setting. Formaldehyde in Q-vax may have led to a greater loss of antigen than heat inactivation. Lastly, 0.49 μM thiomersal in Q-vax may lead to a T helper 2 cell response, thereby reducing IFN-γ production [23].

A major advantage of the IFN-γ production assay is its internal positive and negative control. Consequently, 16.2% of the findings in format A were inconclusive. These were mainly because of low PHA response, suggesting anergy. However, in 76%, PHA did elicit an IFN-γ response, albeit in format B. In the commercial IFN-γ production assays for tuberculosis, indeterminate results are rarely addressed, but low mitogen responses may be as high as 21% [24]. Although inconclusive observations are a limitation of our assay, false-negative or false-positive results are identified, whereas serology and ST lack internal controls.

Because an imperfect gold standard does not allow a realistic estimation of the accuracy of a novel test, we used a Bayesian model (without taking serology and ST as surrogate gold standards). Bayesian models allow the evaluation of the true

accuracy of tests and do not require that any test or combination of tests be perfect [25, 26]. Each unknown parameter in the model should have a prior distribution, based on previous findings. However, data on prevalence of Q fever exposure in the Netherlands are based on serology alone. Moreover, little is known about sensitivity and specificity of serology and STs in Q fever diagnosis. Therefore, we chose to use noninformative priors, which resulted in broad 95% CrI of posterior parameters. With these rough estimates, we found a good performance of the IFN-γ test, similar to the combination of serology and ST.

Specific IFN-γ testing provides data different from serology and ST when used in practice. The discordant results were mainly because of positive IFN-γ production in seronegative and ST-negative individuals. Obviously, the absence of a gold standard makes it impossible to assess definitely whether the IFN-γ assay was more sensitive or less specific than the combination of serology and ST. Bayesian analysis seems to indicate both: somewhat higher sensitivity (87.0% vs 83.0%) and somewhat lower specificity (90.2% vs 95.6%). Moreover, previous studies on immunity to *C. burnetii* after Q fever vaccination, using both lymphocyte proliferation assays and IFN-γ based assays, reveal a higher sensitivity of measurement of cellular immunity than serology [27, 28].

The IFN-γ assay might replace ST in the prevaccination setting because both tests measure cellular immunity to *C. burnetii.* The IFN-γ assay has important operational advantages: the data are available in <2 days, and no follow-up visit is required. Furthermore, the assay can be used repeatedly, whereas ST may affect subsequent (serological) tests by boosting *C. burnetii*–specific immunity [29]. The IFN-γ assay also offers advantages over serology because it is a controlled test with a continuous parameter as read-out, not using titers. In contrast to immunofluorescence serology, there is no inter-reader variability. Moreover, it bypasses the ongoing discussion on phase 1/phase 2 antibodies and optimal cutoff [4, 30, 31]. We are hesitant to state that the IFN-γ production assay could replace serology; we rather see it as being complimentary, with added value in cases with equivocal serology. The additional value in active Q fever disease, both the acute and chronic form, is currently being addressed.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org/).

Notes

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6

A combination of interferon-gamma and interleukin-2 production by *Coxiella burnetii*-stimulated circulating cells discriminates between chronic Q fever and past Q fever

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Abstract

Infection with *Coxiella burnetii* may lead to life-threatening chronic Q fever endocarditis or vascular infections, which are often difficult to diagnose. The present study aims to investigate whether measurement of *in-vitro* interferon-gamma (IFN-γ) production, a key cytokine in the immune response against *C. burnetii*, differentiates chronic from a past cleared infection, and whether measurement of other cytokines would improve the discriminative power. First, *C. burnetii*-specific IFN-γ production was measured in whole blood of 28 definite chronic Q fever patients and compared with 135 individuals with past Q fever (seropositive controls) and 908 seronegative controls. IFN-γ production was significantly higher in chronic Q fever patients than in controls, but with overlapping values between patients and seropositives. Secondly, the production of a series of other cytokines was measured in a subset of patients and controls, which showed that interleukin (IL)-2 production was significantly lower in patients than in seropositive controls. Subsequently, measuring IL-2 in all patients and all controls with substantial IFN-γ production showed that an IFN-γ/IL-2 ratio >11 had a sensitivity and specificity of 79% and 96%, respectively, to diagnose chronic Q fever. This indicates that a high IFN-γ/IL-2 ratio is highly suggestive for chronic Q fever. In an additional group of 25 individuals with persistent high anti-*Coxiella* phase I IgG titres without definite chronic infection, all but six showed an IFN-γ/IL-2 ratio <11. In conclusion, these findings hold promise for the often difficult diagnostic work-up of Q fever and the IFN-γ/IL-2 ratio may be used as an additional diagnostic marker.

Introduction

Q fever is caused by the intracellular bacterium *Coxiella burnetii.* Some 1–5% of the infected individuals present with Q fever endocarditis or infection of an aneurysm or vascular prosthesis ('chronic Q fever'), months to years after primary infection [1,2]. If left untreated, these conditions are often fatal. Timely diagnosis is therefore warranted, and treatment should be started before irreversible damage has occurred. However, diagnosis of Q fever endocarditis or vascular infection is not easy, as culture is cumbersome and specific polymerase-chain reaction (PCR) lacks sensitivity [3,4]. To date, serology is a key factor in early detection of persistent infection, based on measurement of antibodies against the two antigenic forms of *C. burnetii*, phase I and II organisms, with high phase I anti-*C. burnetii* IgG titres (in the absence of acute Q fever) indicative for chronic Q fever [5]. The appropriate cut-off titre that differentiates it from a past cleared infection is debated; the currently proposed cut-offs 1:1024 or 1:1600 have low specificity [5–7].

Appropriate cytokine production is pivotal for the cellular defence against the intracellular bacterium *C. burnetii* [8, 9], with interferon-γ (IFN-γ), derived from T-cells and NK-cells, stimulating macrophage microbicidal activity [10]. IFN-γ is under the control of type I interferons and interleukin-12 (IL-12), IL-18 and IL-23 [11]. Other cytokines also play a role: in Q fever endocarditis, up-regulation of monocyte-derived tumour necrosis factor-*a* (TNF-α), IL-1β and IL-6 from patients has been reported [12], as well as overproduction of IL-10 [13,14]. Benoit *et al*. showed that *C. burnetii* induces M2 polarization of macrophages *in vitro* with up-regulation of IL-1Receptor antagonist (IL-1Ra) and IL-6, and down-regulation of TNF-α [15]. In the adaptive immune response to *C. burnetii*, cytokines such as IL-2, stimulating lymphocyte proliferation and development of memory responses [16], could also be important.

We recently showed that measurement of the cellular immune response (i.e. *in-vitro C. burnetii*-specific IFN-γ production in whole blood) identifies individuals who have been previously exposed to *C. burnetii* [17]. In the present study, we assessed the production of IFN-γ, in combination with other cytokines mentioned above, in patients with chronic Q fever, with the aim of identifying a cytokine profile that may aid in the timely diagnosis of Q fever endocarditis or vascular infection.

Materials and Methods

Ethics statement

The study was approved by the Medical Ethical Committee Arnhem-Nijmegen and written informed consent was obtained from all subjects.

Study population

Twenty-eight Q fever endocarditis or vascular patients were recruited from participating hospitals. At the time of diagnosis, all patients had phase I IgG titres ≥1:1024 (in the absence of acute Q fever), with either a positive *C. burnetii* PCR in serum ($n = 9$) or tissue ($n = 8$) or both ($n = 3$) and/or signs of endocarditis as defined by the modified Duke criteria, or undisputable evidence of vascular (prosthetic) infection on positron emission tomography/computed tomography (PET/ CT)-scan or ultrasound ($n = 8$). Nine were diagnosed with Q fever endocarditis and 19 had a vascular (prosthesis) Q fever infection. They fulfilled the criteria for 'proven chronic Q fever' of the Dutch consensus group on chronic Q fever diagnostics [18].

Individuals screened in the Dutch Q fever vaccination campaign from January to April 2011, as previously described, were used as controls [17]. They all had pre-existing valvular or vascular risk factors for Q fever endocarditis or vascular infection [19]. Control individuals were classified as seronegative if both serological testing and the Q-vax® skin test (CSL, Parkville, Australia) were negative ($n = 908$). Controls were classified as seropositive when serological tests showed anti-*C. burnetii* antibodies (phase I or II IgG ≥1:32) without signs or symptoms of persistent Q fever infection, and without a serological profile suggesting chronic Q fever infection (phase I IgG ≤1:512), more than 1 year after the Q fever epidemic ($n = 135$).

In a second stage of our study, an additional group of 25 patients was included, in whom the diagnosis of chronic *C. burnetii* infection was suspected based on serology, all having persistent phase I IgG ≥1:1,024, but could not be confirmed with PCR, or definite valvular or vascular focus of infection on echocardiography or PET/CT. Fourteen of them had pre-existing valvular or vascular risk factors.

Measurement of *C.burnetii*-specific antibodies and detection of *C. burnetii* DNA

IgG-antibodies against *C. burnetii* phase I and phase II were measured by a commercially available immunofluorescence assay (IFA; Focus Diagnostics, Cypress, CA, USA).

Coxiella burnetii DNA in blood (serum/plasma) and tissue was obtained using real-time PCR targeting the IS1111a insertion element [20].

In-vitro whole blood stimulation

Cytokine production was measured in whole blood stimulation, based on previous findings [17]. Venous blood was drawn into 5-mL endotoxin-free lithium-heparin tubes (Vacutainer, BD Biosciences) and samples were processed within 12 h. Incubation was performed as previously described [17]. *Coxiella burnetii* Nine Mile (NM) RSA 493 phase I [21] was used as well as Q-vax vaccine, containing formaldehyde-inactivated *C. burnetii* Henzerling strain phase I. The mitogen phytohaemagglutinin (PHA, Sigma-Aldrich, St Louis, MO, USA) was used as positive control.

One aliquot was incubated with only culture medium as negative control. After incubation, blood cultures were centrifuged at 4656 *g* for 10 min and supernatants were stored at -20°C until assayed.

Cytokine measurements

IFN-γ concentration was measured in all samples, using a commercial enzyme-linked immunosorbent assay (ELISA; Pelikine compact, Sanquin, Amsterdam, the Netherlands) as previously described [17].

To find out if other cytokines would improve the discrimination between patients and controls, a series of other cytokines was measured in a subset of patients and samples: TNF-α, IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-10 were measured using a multiplex beads assay (Merck Millipore, Billerica, MA, USA). IL-12p70, IL-23 and IL-18 were measured simultaneously in a magnetic beads multiplex assay (Merck Millipore) according to the manufacturer's instructions.

Statistical analysis

Graphpad Prism (Graphpad Software Inc., version 5) was used to analyze the data. Cytokine results were displayed as individual values or expressed as medians with interquartile range. The Mann–Whitney *U*-test was used to determine differences between groups. Spearman's rho correlation coefficient was used to calculate correlation. Receiver operator characteristic (ROC) curves were constructed, and the area under the curve (AUC) was assessed for the accuracy of measuring (a ratio of) cytokine production. $p < 0.05$ was considered significant.

Results

Patients and controls

From January 2011 until January 2012, blood samples were obtained from 28 patients with Q fever endocarditis or vascular infection. Their mean age (± standard deviation) was 66.2 (± 11.8) years; 78.6% were male. At the time of blood sampling, ten patients did not yet receive antibiotics. The median duration of antibiotic therapy among patients on treatment (n = 18) was 7.5 months (range 1–26 months). Patient characteristics at the time of blood sampling are shown in Table 1.

Of the control individuals, 908 individuals (aged 63.6 ± 14.0, 61.6% male) were seronegative; 135 individuals (aged 60.8 ± 15.1, 77.8% male) were seropositive. None of the seropositive controls had detectable IgM antibodies without IgG, suggesting the absence of acute Q fever.

Table 1 Characteristics of 28 patients with definite chronic Q fever

Number	Sex	Age (yr)	Focus of infection	Phase I IgG titre[a,b] (inverse)	Phase II IgG titre[a,b] (inverse)	PCR serum/ plasma[a]	Duration of antibiotic treatment (months)[a]
1	Male	82	Prosthetic valve	8192	16384	Positive	0
2	Male	72	Vascular prosthesis	8192	32768	Positive	0
3	Female	55	Vascular prosthesis	16384	65536	Positive	0
4	Male	69	Valvular repair	16384	16384	Positive	0
5	Male	70	Vascular prosthesis	4096	8192	Negative	0
6	Male	68	Vascular prosthesis	1024	2048	Negative	0
7	Male	68	Vascular prosthesis	32768	16384	Negative	0
8	Male	30	Vascular prosthesis	131072	131072	Positive	0
9	Female	77	Prosthetic valve	32768	32768	Positive	0
10	Male	69	Vascular prosthesis	8192	8192	Negative	0
11	Male	86	Vascular prosthesis	16384	8192	Negative	1
12	Male	66	Prosthetic valve	131072	131072	Positive	1
13	Male	64	Aortic aneurysm	8192	8192	Negative	2
14	Male	58	Prosthetic valve	2048	4096	Negative	6
15	Male	72	Biovalve	2048	2048	Negative	7
16	Female	60	Aortic aneurysm	8192	8192	Negative	7
17	Male	47	Vascular prosthesis	32768	32768	Positive	7
18	Male	73	Vascular prosthesis	8192	8192	Negative	7
19	Female	65	Biovalve	8192	8192	Positive	8
20	Male	72	Vascular prosthesis	8192	4096	Negative	9
21	Male	83	Aortic aneurysm	2048	2048	Negative	13
22	Female	66	Vascular prosthesis	2048	2048	Negative	18
23	Male	58	Vascular prosthesis	8192	32768	Negative	18
24	Male	80	Prosthetic valve	1024	4096	Positive	20
25	Male	69	Aortic aneurysm	4096	4096	Negative	20
26	Male	68	Aortic aneurysm	1024	512	Negative	20
27	Female	56	Vascular prosthesis	2048	2048	Negative	24
28	Male	51	Prosthetic valve	n.a.[c]	n.a.[c]	Negative	26

n.a., not available. [a] At the moment of blood sampling. [b] As measured with immunofluorescence assay (IFA). [c] Not available, complement fixation test (CFT) IgG anti-phase I > 160 and anti-phase II >640.

Interferon-gamma production in 24 h-stimulated whole blood

We measured the IFN-γ production (stimulated–unstimulated) in undiluted whole blood incubated for 24 h with PHA, Q-vax or *C. burnetii*-NM in all patients and all controls (Fig. 1). Aspecific PHA-induced IFN-γ production did not differ between groups. In contrast, both *C. burnetii*-antigens induced significantly more specific IFN-γ in patients (median 151 pg/mL and 2486 pg/mL by Q-vax and *C. burnetii*-NM, respectively) than in seropositive controls (3.0 pg/mL and 120 pg/mL) (p <0.001) and seronegative controls (0.0 and 0.0 pg/mL) (p <0.001). Of interest, longer incubation shows higher mitogen-induced IFN-γ production than *C. burnetii*-specific production (data not shown). Apparently, the specific response is more rapid than the mitogen response.

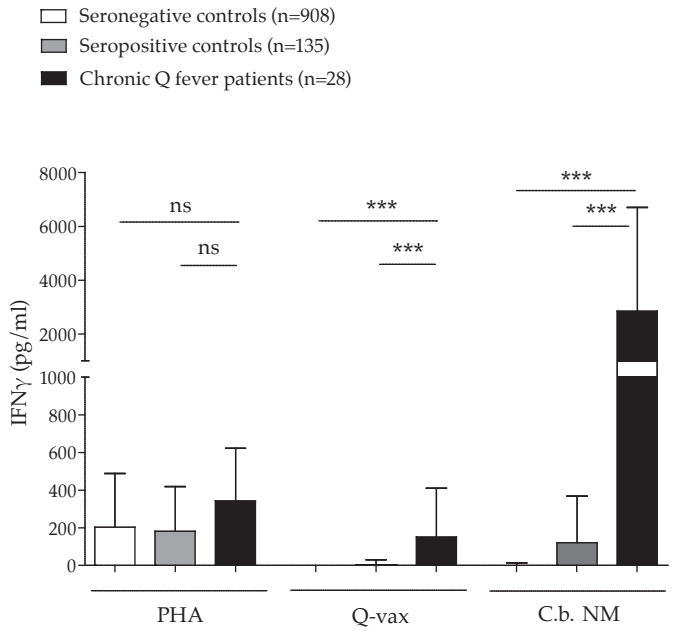


Figure 1. *Coxiella burnetii*-induced IFN-γ production is higher in patients than in seropositive and seronegative controls. Net IFN-γ production (stimulated minus unstimulated) is shown after 24 h incubation of whole blood with phytohaemagglutinin (PHA) or *C. burnetii*-specific antigens (Q-vax and *C.b.*-NM). Median ± IQR are shown. The Mann– Whitney *U*-test was used to compare medians. *C.b.*-NM, *C. burnetii* Nine Mile; ns = not significant, ***p <0.001.

ROC curves for *C. burnetii*-induced IFN-γ for patients vs. seropositive controls showed an accuracy (AUC) of 0.8664 (95% CI, 0.7933–0.9395; p <0.0001) (Fig. 2a); Q-vax-stimulated IFN-γ production showed lower accuracy (0.8484; 95% CI,

0.7639–0.9330; p <0.0001) (Figure S1). Therefore, further analyses were performed only with *C. burnetii*-NM-stimulated samples. There was a considerable overlap between IFN-γ production in seropositive controls and Q fever patients (Fig. 2b). Choosing 500 pg/mL as the optimal cut-off, 75.0% (21/28) of the patients and 17.8% (24/135) of the seropositive individuals had a value above this cut-off.

Interestingly, the height of IgG anti-phase I antibody titre was significantly correlated with the amount of IFN-γ produced. Spearman's rho in the total of seropositive controls and Q fever patients was 0.3069 (p <0.001).

Further analyses of IFN-*γ* production did not show significant differences between untreated (*n* = 10) and treated (*n* = 18) Q fever patients (p 0.11); duration of treatment (in months) did not correlate with IFN-*γ* production (p 0.37).

Cytokine profiles in 48-h *C. burnetii* Nine Mile-stimulated diluted blood in a subset of patients and controls

To find out whether measurement of one or more additional cytokines would help to distinguish chronic Q fever from past infection, both conditions showing high IFN-*γ* production, we measured the production of TNF-*α*, IL-1*β*, IL-1Ra, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-23, IL-18 and IL-2. Because not all cytokines were expected to be detectable in the first 24 h, 48-h *C. burnetii*-NM-stimulated diluted blood samples were used. Cytokine production in samples of 16 patients (obtained in the first 10 months of the study), 18 seropositive controls with substantial (≥32 pg/mL) IFN-*γ* production in 24 h and 18 seronegative controls with low IFN-*γ* production, were compared. The results for IFN-*γ*, IL-2, IL-1*β*, TNF-*α*, IL-10 and IL-1Ra are shown as Figure S2 in the supporting information. As expected, the median IFN-*γ* production did not differ significantly between patients and the selected seropositive individuals. Most interestingly, production of IL-2 was significantly lower in patients (median 5.0 pg/mL) than in these seropositive controls (median 39.5 pg/mL) (p <0.001).

The production of the monocyte-derived cytokines IL-1*β* and TNF-*α* was high in both chronic Q fever patients and in seropositive individuals, but not significantly different (p 0.24 and p 0.16 for IL-1*β* and TNF-*α*, respectively). However, both were significantly higher than in seronegative controls. The same pattern was seen for IL-6 (not shown).

The anti-inflammatory cytokine IL-10 production also did not differ between patients and seropositive controls. However, patients had significantly higher IL-10 production than seronegative individuals (p <0.01). IL-1Ra did not differ between groups.

The *C. burnetii*-NM-stimulated production of IL-4, IL-5, IL-18 and IL-23 remained below detection limit. IL-12p70 levels were very low and did not discriminate.

IFN-γ/IL-2 ratio for diagnosis of chronic Q fever

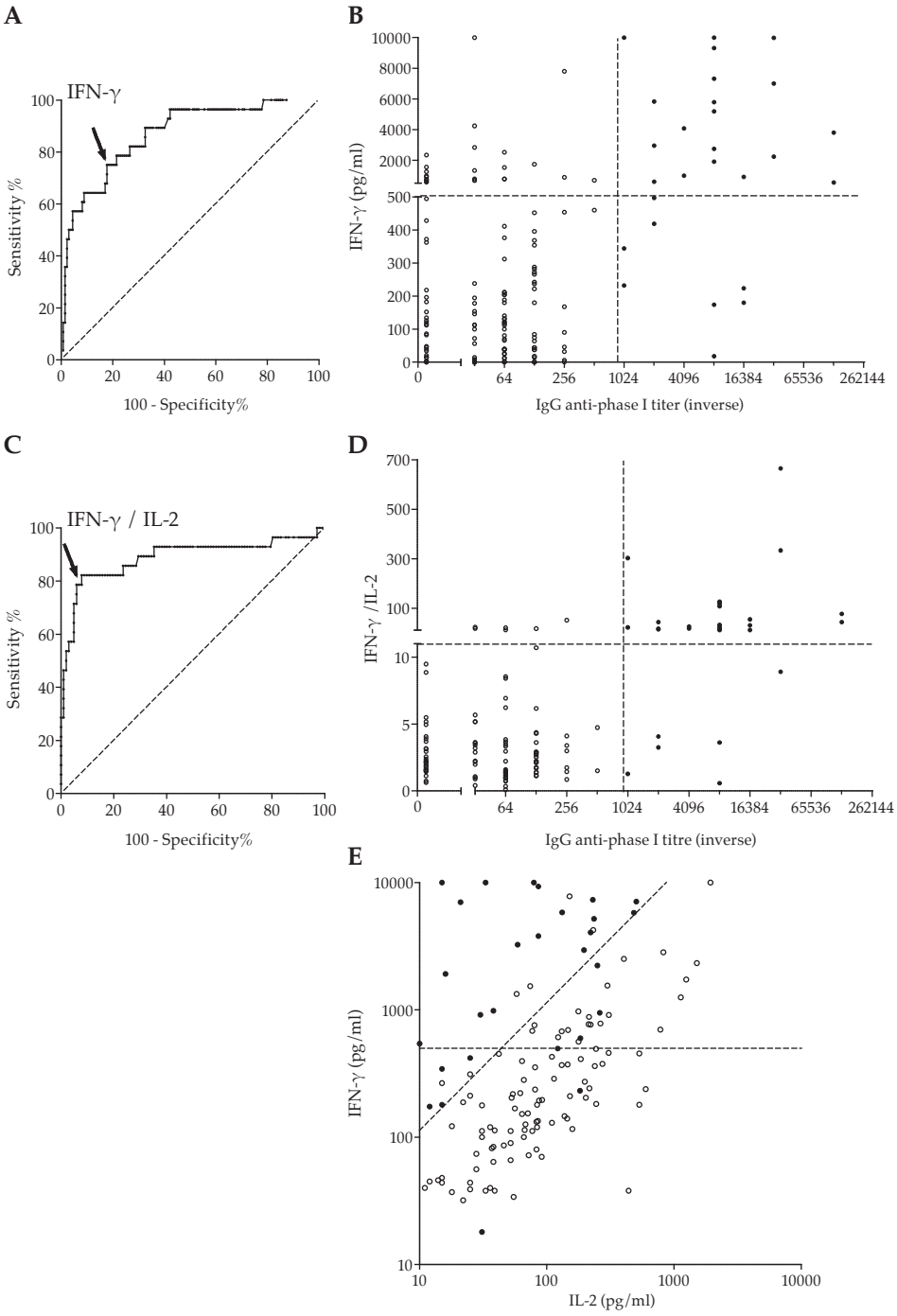
In previous subset, patients with chronic Q fever showed high IFN-γ and low IL-2 production, whereas seropositive controls with high IFN-γ production showed high IL-2.

Next, we assessed IL-2 production in all patients and all 102 seropositive controls with substantial IFN-γ production ≥32 pg/mL. The IFN-γ/IL-2 ratio was calculated for each individual (Fig. 2d). The AUC of the ROC-curve for patients vs. seropositive controls was 0.8873 (95% CI, 0.7983–0.9762; p <0.0001) (Fig. 2c). In 22/28 (78.6%) patients, the ratio was above 11, compared with only 6/102 (5.9%) seropositive controls with an IFN-γ production ≥32 pg/mL and 6/135 (4.4%) of all seropositives. So, the number of high values in the seropositive controls diminished by 75% (from 24 to 6) at cut-off 11 for IFN-γ/IL-2 ratio compared with the cut-off 500 pg/mL for IFN-γ production alone (Fig. 2e).

The IFN-γ/IL-2 ratio did not correlate with the duration of antibiotic treatment in patients (p 0.44), neither was there a significant difference between treated and untreated patients (p 0.55). However, patients with positive *C. burnetii* PCR in serum or plasma at the moment of blood sampling (n = 10), had a significantly higher IFN-γ/IL-2 ratio than those with negative PCR (n = 18) (median IFN-γ/IL-2 ratio of 92.9 and 15.9, respectively, p <0.01).

IFN-γ/IL-2 ratio in individuals with high phase I IgG without definite chronic Q fever

Subsequently, the IFN-γ/IL-2 ratio was determined in the group of 25 individuals (aged 63.7 ± 13.9, 72.0% male) in whom chronic Q fever was suspected based on persistent high IgG anti-*C. burnetii* phase I ≥1:1024, but without definite diagnosis of chronic *C. burnetii* infection based on PCR, echocardiography or PET/CT-scan. The results are shown separately for those with and those without pre-existing cardiovascular risk factors for chronic infection (Fig. 3). All but six had an IFN-γ/IL-2 ratio below the cut-off. A high IFN-γ/IL-2 ratio was found in 2/11 (18%) in the group without and 4/14 (29%) in the group with risk factors. The details of these six patients are shown in Table 2. Interestingly, patient 1, without pre-existing risk factors but persistent high phase I IgG and a high IFN-γ/IL-2 ratio of 39, was a veterinarian with non-specific complaints and a newly diagnosed cardiac murmur. Echocardiography and PET/CT-scan were normal and PCR on serum negative. After discontinuation of 1.5 years treatment with doxycycline and hydroxychloroquine without decreasing antibody titres, titres rose again. It cannot be excluded that this veterinarian experienced ongoing exposure to *C. burnetii*. Patient 2, having no valvular or vascular risk factors for chronic Q fever but persistent high phase I IgG and IFN-γ/IL-2 above the cut-off, was immunocompromised (prednisone use after kidney transplantation). He was started on antibiotics because of suspected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D
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(Previous page) Figure 2. IFN-γ production discriminates between patients with chronic Q fever and seropositive controls, but the ratio of IFN-γ and IL-2 production discriminates better. (a) Receiver operator characteristics (ROC) curve of the *Coxiella burnetii*-NM-stimulated IFN-γ production after 24 h in whole blood of chronic Q fever patients vs seropositive controls. The area under the curve (AUC) is 0.8664 (95% CI, 0.7933–0.9395; p <0.0001). The arrow indicates the optimal cut-off of IFN-γ production (500 pg/mL) with best combination of sensitivity and specificity. (b) The individual IFN-γ production is shown for chronic Q fever patients (*n* = 28) and seropositive controls (*n* = 135), plotted against phase I IgG titres. Each dot indicates one individual. The horizontal line indicates the cut-off at 500 pg/ml. The vertical line distinguishes patients (closed circles) from controls (open circles). (c) ROC curve of the IFN-γ/IL-2 ratio in Q fever patients vs seropositive controls. AUC is 0.8873 (95% CI, 0.7983–0.9762). The arrow indicates the optimal cut-off of IFN-γ/IL-2 ratio at 11. (d) The individual IFN-γ/IL-2 ratio is shown for chronic Q fever patients (*n* = 28) and seropositive controls with IFN-γ production ≥32 pg/mL (*n* = 102), plotted against phase I IgG. Each dot indicates one individual. The horizontal line indicates an IFN-γ/IL-2 ratio cut-off at 11. The vertical line distinguishes patients (closed circles) from controls (open circles). (e) Comparison between IFN-γ alone and the ratio of IFN-γ/IL-2 to identify patients. IFN-γ plotted against IL-2 production shows patients (black dots) and seropositive controls (open dots). All patients (*n* = 28) and all seropositive controls with IFN-γ production ≥32 pg/mL (*n* = 102) were included. The horizontal line indicates the cut-off of IFN-γ at 500 pg/mL. The sloping line indicates the cut-off IFN-γ/IL-2 ratio at 11.

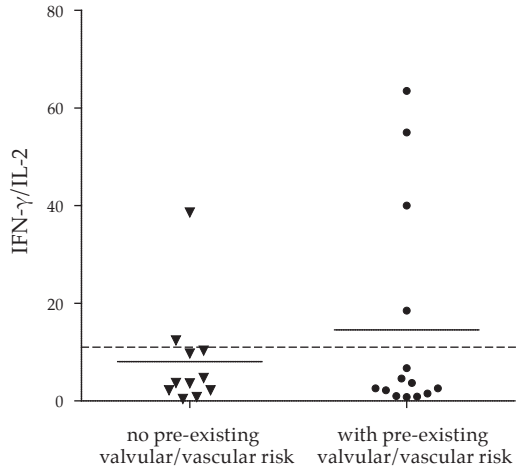

Figure 3. The ratio of IFN-γ and IL-2 production in individuals with high phase I IgG, without definite chronic Q fever. These 25 individuals were diagnosed with persistent phase I IgG ≥1:1024, but no definite chronic Q fever based on PCR, echocardiography or PET/CT-scan. Eleven had no pre-existing cardiovascular risk factors for chronic Q fever, while the other 14 had pre-existing valvular or vascular defects. The horizontal dotted line indicates the previous established cut-off IFN-γ/IL-2 ratio at 11.

Table 2 Characteristics of six individuals with high IFN-γ/IL-2 ratio and persistent high phase I IgG without definite chronic Q fever

Case number, sex, age (yr)	Pre-existing valvular or vascular risk factor(s)	Serology at first diagnosis of persistent high phase I IgG (phase I IgG/ phase II IgG; inverse)	Results of PET/CT scan and echocardiography	Long-term (1.5–2 years) antibiotic treatment	Serology at moment of blood sampling (phase I IgG/ phase II IgG; inverse)	IFN-γ/ IL-2 ratio at moment of blood sampling
1, Female, 41	None	1024/1024	PET/CT and echocardiography negative	Yes	1024/1024	39
2, Male, 67 [a]	None	8192/8192	PET/CT suspect aspect located at mitral valve, not conclusive for endocarditis; TEE negative	Yes	8192/8192	12
3, Male, 71	Prosthesis aorta; fem-fem crossover bypass	16384/16384	PET/CT possible focus of infection at fem-fem crossover bypass, not conclusive, could be reactivity to operation; TEE negative	Yes	4096/4096	55
4, Male, 64	Prosthesis aorta	1024/4096	PET/CT negative	No	512/1024	40
5, Male, 71	Mitral and tricuspidal valve repair	4096/2048	Echocardiography negative	Yes	4096/4096	19
6, Male, 37	Mechanical aortic valve prosthesis	32768/16384	PET/CT and echocardiography negative	Yes	4096/4096	63

PET/CT, positron emission tomography/computed tomography; TEE, transoesophageal echocardiography; IFN-γ, interferon-gamma; IL-2, interleukin-2.
[a] This patient was immunocompromised (prednisone use after kidney transplantation).

mitral valve involvement based on PET/CT, which was considered inconclusive for endocarditis by the nuclear medicine physician and could not be confirmed by echocardiography. Likewise, patient 3 was treated for suspected Q fever vascular infection; however, the PET/CT was interpreted to be inconclusive because of diffuse uptake in the recently inserted vascular prosthesis. Patients 5 and 6 had cardiac predisposition but no evidence of persistent infection based on PCR, echocardiography or PET/CT-scan. Both, however, were started on long-term antibiotics because of the high risk of Q fever endocarditis. Only patient 4 did not receive antibiotics and had spontaneously decreasing phase I IgG titre, which persisted at 1:512 without signs or symptoms of chronic active infection.

Discussion

In the present study, we show that Q fever endocarditis or vascular patients exhibit high *in-vitro* IFN-γ production and low IL-2 production, while individuals with a past infection showed both high IFN-γ and high IL-2 production. The production of monocyte-derived pro-inflammatory cytokines IL-1β, TNF-α and IL-6 was high in patients as well as in individuals with past infection and did not discriminate. Similarly, anti-inflammatory cytokines IL-10 and IL-1Ra were not significantly different in Q fever patients and individuals with a past infection. In this population, the IFN-γ/IL-2 production ratio >11 had a sensitivity and specificity of 79% and 96%, respectively, to diagnose chronic Q fever.

Interestingly, we found that Q fever endocarditis and vascular patients display high *C. burnetii*-specific IFN-γ production in whole blood, while it is commonly assumed that chronicity of Q fever infection is due to T-lymphocyte unresponsiveness and impaired IFN-γ production [22,23]. We show here that *C. burnetii*-specific IFN-γ production in these patients is even higher than in individuals with past *C. burnetii* infection. We obtained the same results when using isolated peripheral blood mononuclear cells from chronic patients and controls (unpublished data). This is in accordance with the findings of Limonard *et al.* [24] reporting higher numbers of IFN-γ-positive cells in three chronic Q fever patients compared with nine convalescent controls, using a *Coxiella* ELISPOT assay. Apparently, this high IFN-γ production by peripheral blood cells is not sufficient to activate *C. burnetii*-infected monocytes/macrophages at the site of infection, to such an extent that the infection is cleared. We hypothesize that downstream of IFN-γ, the response to this cytokine is aberrant in patients with persistent *C. burnetii* infection.

The measurement of the production of monocyte-derived cytokines did not aid in differentiation between chronic active Q fever patients and individuals previously exposed to *C. burnetii*. Nevertheless, both groups had higher production

than naive controls. From this it can be concluded that restimulation with *C. burnetii* in primed individuals leads to enhanced production of cytokines by monocytes. With regard to the production of anti-inflammatory cytokines in chronic Q fever patients, only that of IL-10 was higher than in seronegative controls. This is in line with previous reports on patients with Q fever endocarditis [13,14]. The latter studies also reported low IL-10 in individuals with Q fever without chronic evolution. IL-10 appeared to induce *C. burnetii* replication and its neutralization inhibits bacterial replication in monocytes from patients with Q fever endocarditis [25]. Thus, IL-10 may be important in the development of chronic Q fever, but we found that measurement of *C. burnetii*-specific IL-10 production is not a useful diagnostic marker.

Our finding that IL-2 production was significantly lower in chronic Q fever patients than in seropositive controls may be to do with the types of T cell involved in the *C. burnetii*-specific immune responses. In chronic infection, we assume that increased numbers of circulating *C. burnetii*-specific effector T cells produce IFN-γ and low amounts of IL-2 upon activation. In seropositive controls, it is probably the central memory T cell that dominates and produces mainly IL-2 [26]. We showed that the ratio of IFN-γ to IL-2 production is more specific than IFN-γ production alone (96% vs 82%), and has a slightly higher sensitivity (79% vs 75%) to distinguish patients from seropositive controls. The relative high specificity of the IFN-γ/IL-2 ratio suggests that seropositive individuals with a ratio above the cut-off merit thorough follow-up for the progression to Q fever endocarditis or vascular infection.

In tuberculosis, measurement of specific IL-2 production in addition to IFN-γ also helps to differentiate between active and latent infection. Here too, a high specific IFN-γ and low IL-2 production is found in active infection, and both high IFN-γ and high IL-2 production indicate latent tuberculosis [27,28]. A shift from T cells secreting only IFN-γ and IFN-γ/IL-2 to T cells secreting IFN-γ/IL-2 and only IL-2 is reported during treatment of active tuberculosis [29].

Ten of the 28 patients were not yet on antibiotic treatment at the moment of blood sampling. The absence of a significant difference in cytokine profile between untreated and treated patients suggests that the cytokine profile observed is not affected by the use of antibiotics. Still, the IFN-γ/IL-2 ratio seems to be related to the load of *C. burnetii*, because patients with positive PCR for *C. burnetii* DNA in serum or plasma have a significantly higher ratio than patients with negative PCR. Longitudinal studies are needed to assess the applicability of the IFN-γ/IL-2 ratio in follow-up during treatment of patients with chronic Q fever.

All 28 patients included in the first part of the study to evaluate the performance of cytokine(s) production measurements, had definite Q fever endocarditis or vascular infection. We subsequently performed the measurements of IFN-γ/IL-2 in a group of 25 individuals with an uncertain diagnosis (i.e. having high phase I

IgG without definite diagnosis of chronic *C. burnetii* infection based on clinical criteria, PCR or definite focus of infection on diagnostic imaging). It shows the difficulties in assessing the value of a new marker in a group of patients with uncertain diagnosis, some of whom are highly suspected of having chronic Q fever, but not fulfilling the criteria for definite chronic Q fever, neither those of Raoult *et al.* nor those of the Dutch consensus [18,30]. In the great majority of these difficult cases (19/25), the IFN-γ/IL-2 ratio was low. This may imply absence of chronic *C. burnetii* infection or a more low-grade infection. In some, the diagnosis remains unclear because PCR and imaging techniques may have lacked sensitivity to make a definite diagnosis before long-term antibiotic treatment was started.

In conclusion, we found that a high IFN-γ/IL-2 ratio is highly suggestive for chronic Q fever. This finding holds promise for the often difficult diagnostic work-up of Q fever patients and may be used as an additional diagnostic marker.

Acknowledgements

We thank Hendrik-Jan Roest (Central Veterinary Institute, Lelystad, the Netherlands) for providing the *C. burnetii* Nine Mile antigens. These data were presented on a poster at the 22nd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) in April 2012 in London.

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Supplementary Figures

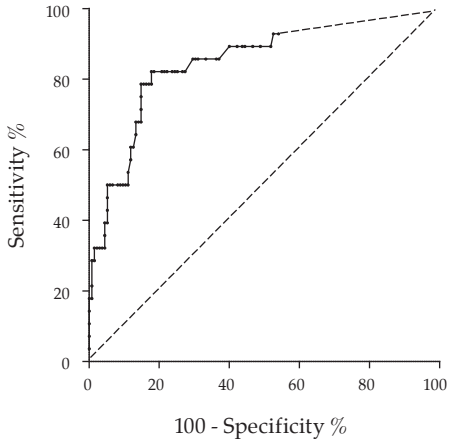
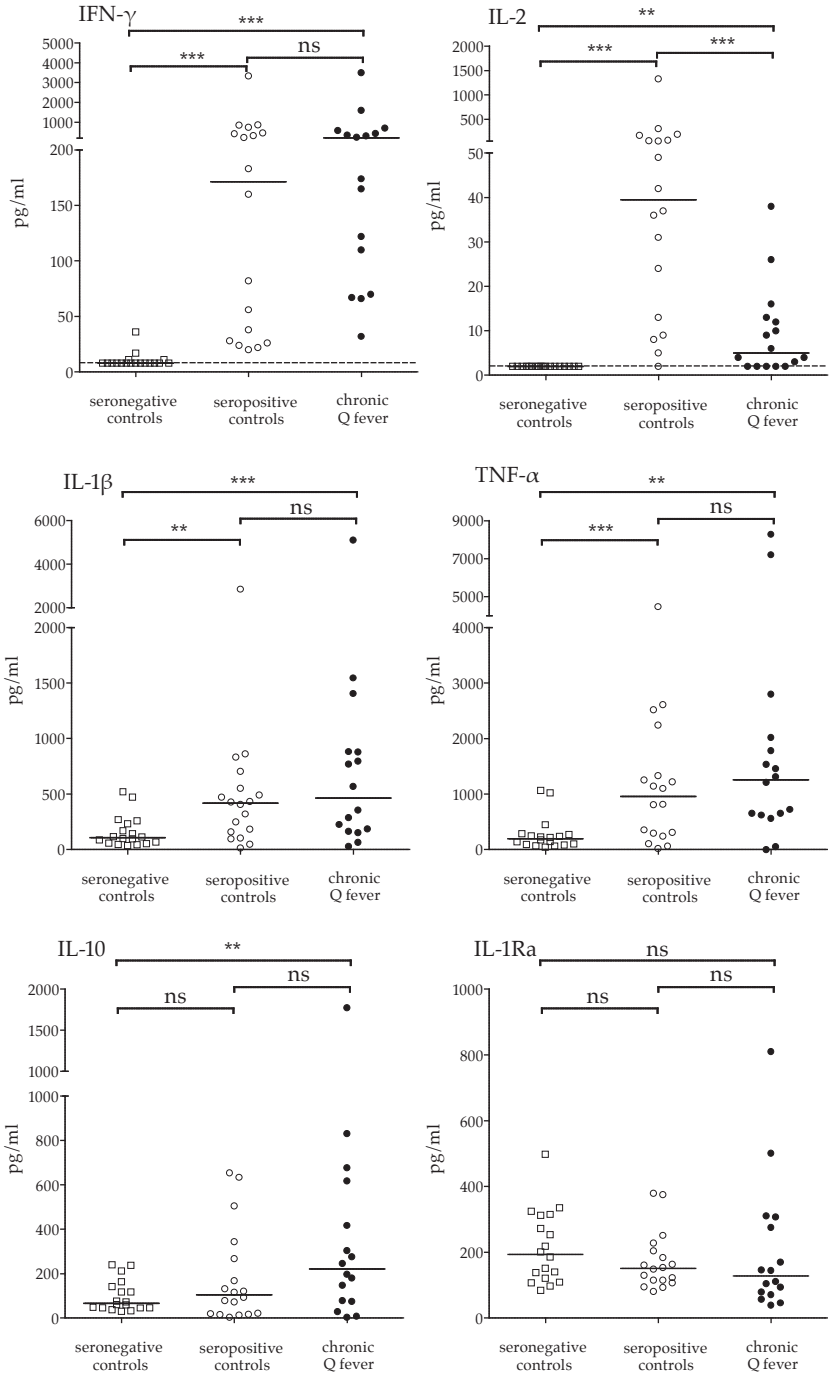


Figure S1. Receiver operator characteristics (ROC) curve of the Q-vax stimulated IFN-γ production after 24 hours in whole blood of chronic active Q fever patients versus seropositive controls. The Area Under the Curve (AUC) is 0,8484 (95% CI; 0.7639-0.9330, P<0.0001).

(Next page) Figure S2. *C. burnetii* induced cytokine profile in patients with chronic Q fever, compared to seronegative and seropositive controls. Cytokines interferon (IFN)-γ, interleukin (IL)-2, IL-1β, tumor necrosis factor (TNF)-α, IL-10 and IL-1Receptor antagonist (IL-1Ra) in 48 hours *C. burnetii*-NM stimulated blood are shown for a selection of seronegatives (n=18), seropositives (n=18) and Q fever endocarditis or vascular patients (n=16). Horizontal lines indicate the median value of the respective population. The dotted line represents the lower detection limit of the assay. Mann-Whitney U test was used to compare medians. ns = not significant, * P<0.05, **P<0.01, *** P<0.001.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7

Specific in vitro interferon-gamma and IL-2 production as biomarkers during treatment of chronic Q fever

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Abstract

Background: Antibiotic treatment of chronic Q fever is cumbersome and of long duration.To monitor treatment, there is a need for alternative biomarkers. *Coxiella burnetii*-specific interferon (IFN)-γ and interleukin (IL)-2 production reflect the type of effector and memory T-cell response. In chronic Q fever, *C. burnetii*-specific IFN-γ production is higher and IL-2 production is lower than in individuals with past Q fever. Here we explore whether *C. burnetii*-specific IFN-γ and IL-2 production correlate to treatment response.

Methods: We studied the longitudinal *C. burnetii*-specific IFN-γ/IL-2 ratio in fifteen proven chronic Q fever patients. All patients were followed for at least 18 months during antibiotic treatment. Treatment was considered successful when clinical recovery was observed, a positive PCR for *C. burnetii* DNA in blood became persistently negative, anti-phase I IgG showed a fourfold decrease or more, and imaging techniques showed disappearance of infectious foci.

Results: Overall, the IFN-γ/IL-2 ratio declined when patients experienced a successful treatment outcome. When treatment failed, IFN-γ/IL-2 ratios did not significantly decrease. The median (±IQR) slope of the longitudinal IFN-γ/IL-2 ratio with successful treatment was −2.10 (−7.02 to −0.06), and −0.15 (−1.13 to 0.25) with unsuccessful treatment ($P = 0.19$). Q fever endocarditis patients had higher IFN-γ/IL-2 ratios than patients with endovascular infections.

Conclusion: We propose that the IFN-γ/IL-2 ratio can be used as an additional biomarker for monitoring chronic Q fever treatment, with declining ratios being indicative of successful treatment.

Introduction

Q fever is a zoonosis, caused by the Gram-negative, intracellular bacterium *Coxiella burnetii* (Raoult et al., 2005; Parker et al., 2006). Following primary infection, 1–5% of patients develop chronic infection, which can become clinically overt months to years later (Maurin and Raoult, 1999). Endocarditis and infection of a vascular aneurysm or prosthesis are the most common manifestations of chronic Q fever (Botelho-Nevers et al., 2007; Million et al., 2010). Pre-existent cardiac valvular abnormalities, aortic aneurysms, vascular grafts, and immune-compromised state are risk factors (Raoult et al., 2000; Fenollar et al., 2001; Landais et al., 2007).

Clinical symptoms of chronic Q fever are often non-specific, and the diagnosis relies on identifying pre-existing risk-factors, the results of anti-*C. burnetii* serology and PCR for *C. burnetii* DNA on blood or tissue, and results of imaging techniques (Wegdam-Blans et al., 2012a; Anderson et al., 2013). Untreated chronic infection leads to severe morbidity, with a mortality up to 60% (Million et al., 2010). Long-term antibiotics, preferably doxycycline combined with hydroxychloroquine (Raoult et al., 1999; Kersh, 2013), are required to eliminate *C. burnetii*. Antibiotics should be administered for at least 18 months or, in case of a valvular/vascular prosthesis, for at least 24 months (Million et al., 2010). Surgical intervention to replace an infected vascular aneurysm/graft or cardiac valve is often necessary, either in the acute situation of a symptomatic aortic aneurysm or heart failure, or when a patient does not improve on antibiotics (Botelho-Nevers et al., 2007; Kampschreur et al., 2012; Wegdam-Blans et al., 2012b).

The discontinuation of antimicrobial therapy strongly depends on the results of follow-up imaging. In case of vascular infection focus, ^{18}F-fluorodeoxyglucose Positron Emission Tomography/Computed Tomography (FDG-PET/CT) is preferred, which has high sensitivity and specificity for low-grade vascular infections (Merhej et al., 2012; Barten et al., 2013). In cases of Q fever endocarditis, vegetations are difficult to detect on echocardiography, and a negative echocardiogram does not rule out endocarditis (Maurin and Raoult, 1999). Therefore, serology is an important tool during the antibiotic treatment of chronic Q fever. Chronic infection is characterized by high titres of anti-phase I IgG anti-bodies. It is assumed that antibiotic treatment should be continued until these titres have declined at least fourfold, or until titres are below 1:800 in immunofluorescence assay [IFA, or below 1:1024 in a commercial available IFA (Focus Diagnostics)]; (Million et al., 2010). In daily practice, the slow serological decline requires longer treatment, and titres often remain above 1:800 (or above 1:1024 respectively) for a prolonged time.

These limitations show the need for additional biomarkers to monitor treatment of chronic Q fever. In this respect, laboratory tests measuring cell-mediated immune

responses may be of value. Interferon-gamma (IFN-γ) plays a pivotal role in the immune response against the intracellular *C. burnetii* (Dellacasagrande et al., 1999; Andoh et al., 2007). Analogous to IFN-γ release assays (IGRAs) that are widely used in *Mycobacterium tuberculosis* infection (Pai et al., 2004), we previously used whole-blood assays to show that the *C. burnetii*-specific IFN-γ production is significantly increased in people that have been exposed to *C. burnetii* (Schoffelen et al., 2013). The interpretation of IFN-γ production in chronic Q fever is complex, since IFN-γ production is a marker of both immunity and infection. We demonstrated, by measuring a broad panel of cytokines, that *ex-vivo C. burnetii*-specific IFN-γ production is higher and interleukin (IL)-2 production is lower in chronic Q fever patients than in patients with past Q fever (Schoffelen et al., 2014), and concluded that a high IFN-γ/IL-2 ratio has a high specificity to discriminate between these two groups.

The present study follows the hypothesis that the IFN-γ/IL-2 ratio will decline during effective treatment of chronic Q fever. To test this, we followed 15 chronic Q fever patients for at least 18 months during antibiotic treatment and performed whole-blood stimulation assays with measurement of IFN-γ and IL-2 on a regularly basis. This study is the first evaluation of a cell-mediated immunity biomarker for treatment of chronic Q fever.

Materials and Methods

Patients and follow-up

Fifteen chronic Q fever patients, recruited from participating hospitals, were followed in this study for at least 18 months. The study was approved by the Medical Ethical Committee Arnhem-Nijmegen and written informed consent was obtained from all subjects. At the time of diagnosis, all patients had a positive PCR in blood, serum and/or tissue, and anti-phase I IgG titres ≥ 1:1024 (in the absence of acute Q fever). Four patients were diagnosed with endocarditis according to the modified Duke criteria ($n = 4$), and eleven had vascular infection. All fulfilled the criteria of 'proven chronic Q fever' of the Dutch consensus group on chronic Q fever (Wegdam-Blans et al., 2012a). Patients were included at different time points after start of treatment. The start of antibiotic treatment was designated $t = 0$. The three patients that were included at the start of treatment or within 2 months after start of treatment are described in more detail. Treatment was considered successful when clinical recovery was observed, a positive PCR for *C. burnetii* DNA on blood became persistently negative, antiphase I IgG showed a fourfold decrease or more (related to the maximum titre), and imaging techniques showed disappearance of any (vascular or valvular) infectious foci.

Whole blood incubation

Venous blood drawn into 5 mL endotoxin-free lithium-heparin tubes (Vacutainer, BD Biosciences) was processed within 12 h. Blood was aliquoted in separate tubes and incubated at 37°C for 24 h with heat-inactivated *C. burnetii* Nine Mile (NM) RSA493 phase I (Seshadri et al., 2003), mitogen (positive control) or without (negative control).

C. burnetii NM was used in an end-concentration of 10^7 bacteria/mL. Bacteria were cultured in a BSL-3 facility at the Central Veterinary Institute (Lelystad, the Netherlands) as previously described (Schoffelen et al., 2013) and kindly provided by Dr. H. J. Roest. The same batch was used for all assays. The mitogen phytohemagglutinin (PHA, Sigma-Aldrich, St. Louis, MO, USA; 10 μg/mL) was used as a positive control. After incubation, blood cultures were centrifuged at 4656 *g* for 10 min and supernatants were stored at –20°C until assayed.

Cytokine measurements

Interferon-γ concentration in supernatants was measured using a commercial enzyme-linked immunosorbent assay (ELISA; Pelikine compact, Sanquin, Amsterdam, the Netherlands) as previously described (Schoffelen et al., 2014). The background IFN-γ response of the negative control aliquot was subtracted from the stimulated aliquots for each individual sample. In all negative control aliquots, the highest IFN-γ concentration was 46 pg/mL, which was considered acceptable. In addition, all samples showed a net IFN-γ production >24 pg/mL in either the positive control aliquot or in the *C. burnetii*-stimulated aliquot. Thus, all samples were considered valid. IL-2 concentrations in supernatant of the *C. burnetii*-stimulated aliquots were measured using luminex magnetic beads assay (Merck Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

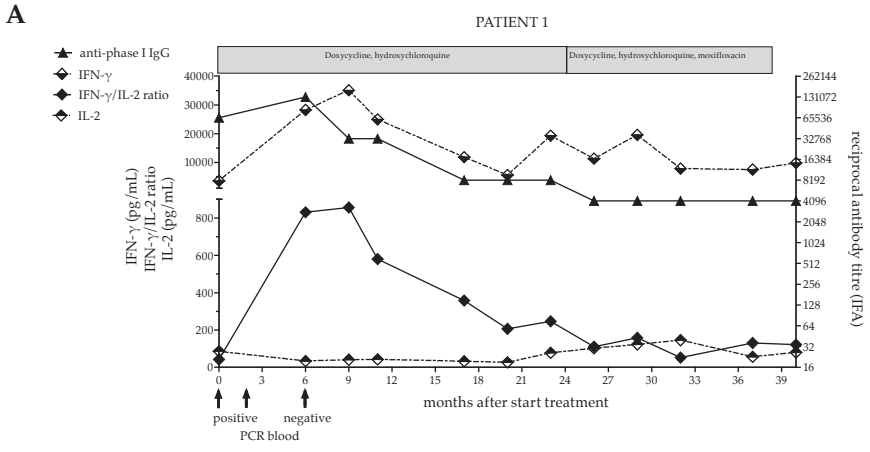
Analysis

Graphpad Prism (Graphpad software Inc., version 5) was used to make the graphs and to analyze the data. Non-linear regression to a straight line with least square fit was performed to obtain the best-fit slope of the IFN-γ/IL-2 ratio of each patient over time. Median ± interquartile range (IQR) slope was compared between groups.

Results

Case 1: a patient with *C. burnetii*-infected vascular prosthesis with successful treatment

A 30-years-old man with a medical history of traumatic rupture of the thoracic aorta, for which he had undergone vascular surgery receiving an endoprosthesis, suffered from new symptoms of malaise, night sweats, weight loss, and chills. Because of the preceding Q fever epidemic, serology for *C. burnetii* was performed, revealing chronic infection with very high IgG titers against phase I and II (both 1:65536). PCR on serum for *C. burnetii* DNA was positive. He was not aware of a preceding acute Q fever episode. FDG-PET/CT showed no signs of infection at the thoracic aortic prosthesis, nor elsewhere. Transesophageal echocardiogram (TEE) showed mild aortic valve insufficiency without vegetations. Although infection of the vascular prosthesis could not be detected with FDG-PET/CT, it was considered the most likely focus of infection. The patient started antimicrobial therapy with doxycycline and hydroxychloroquine after which he made a quick clinical recovery. PCR in serum became permanently negative 5 months after start of therapy. Although anti-phase I IgG titers had decreased from 1:131072 to 1:8192 in 24 months, titers did not decrease further and moxifloxacin was added to the therapy. This regimen was continued for another 14 months, after which it was decided to stop treatment and continue follow-up 3-monthly. The follow-up –6 months so far– was uneventful. This patient, having a good response on the antimicrobial therapy, showed peaking of the IFN-γ/IL-2 ratio during the first 9 months. Thereafter, the ratio declined and was stable at lower values during the last phase of the treatment, analog to the anti-phase I antibody titers (Figure 1A).



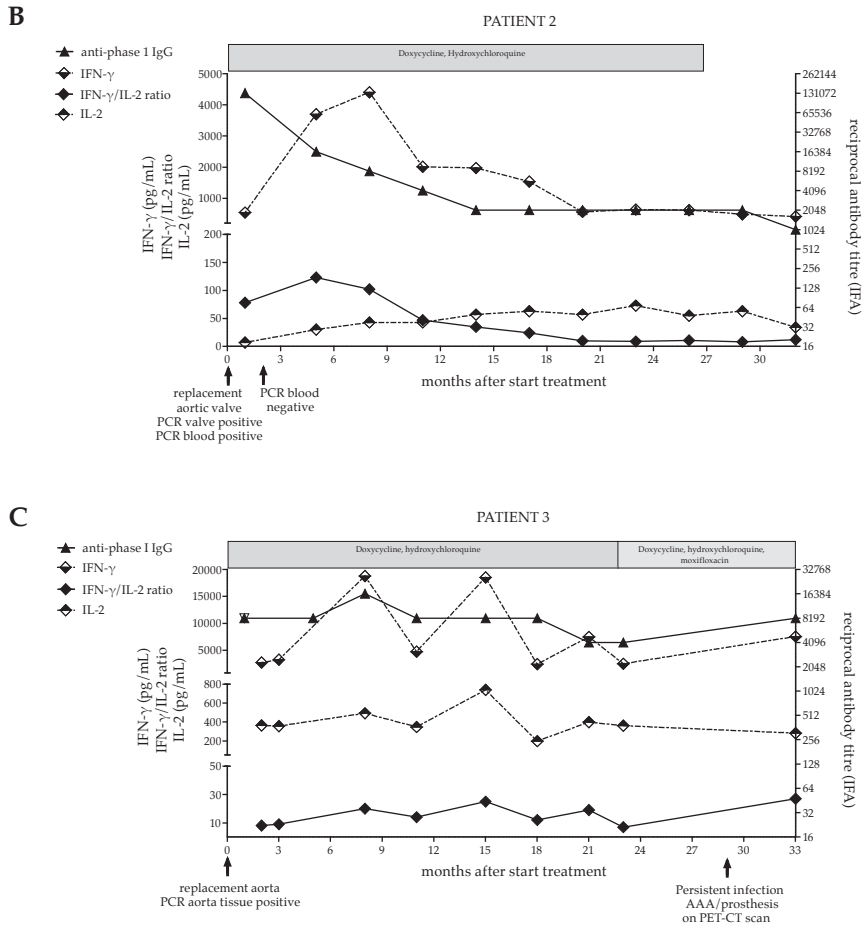


Figure 1. Detailed overview of immunological parameters in (A) patient 1 (B) patient 2, and (C) patient 3 from start of antibiotic treatment to end of study follow-up. The IFN-γ and IL-2 concentrations (dotted lines) and the IFN-γ/IL-2 ratio (black diamant) are shown on the left *y*-axis, the anti-phase I IgG antibody-titer (black triangle) is shown on the right *y*-axis. The course of antibiotic treatment is depicted above each graph.

Case 2: a patient with *C. burnetii* endocarditis with successful treatment

A 66-years-old man had a history of aortic valve stenosis with valvular replacement (homograft) 11 years before. He presented with symptoms of cardiac failure, for which a TEE was performed, which showed vegetations on the valvular prosthesis. Screening for *C. burnetii* revealed chronic Q fever infection with anti-phase I and II

Table 1 Clinical features of the chronic Q fever patients

Nr	Sex	Age (yrs)[a]	Focus of infection	IgG anti-phase I titre[a,b]	IgG anti-phase II titre[a,b]	PCR serum/ plasma[a]	PCR tissue	Duration of antibiotic treatment (months)[c]
1	Male	30	Vascular graft	65536	65536	Pos	n.a.	0
2	Male	66	Aortic valve	131072	131072	Pos	Pos	1
3	Male	64	Aortic aneurysm	8192	8192	Neg	Pos	2
4	Male	46	Vascular graft	65536	131072	Pos	n.a.	7
5	Female	64	Aortic aneurysm	8192	8192	Pos	n.a.	18
6	Male	72	Vascular graft	16384	8192	Neg	Pos	9
7	Male	74	Aortic aneurysm	65536	32768	Neg	Pos	4
8	Female	54	Vascular graft	4096	4096	Neg	Pos	24
9	Male	79	Prosthetic aortic valve	4096	4096	Pos	n.a.	20
10	Male	58	Mitral valve	4096	8192	Pos	Pos	6
11	Male	67	Vascular graft	4096	4096	Neg	Pos	20
12	Female	65	Prosthetic aortic biovalve	8192	16384	Pos	n.a.	8
13	Male	71	Vascular graft	2048	4096	Pos	Pos	35
14	Male	59	Vascular graft	16384	8192	Pos	Pos	29
15	Male	74	Vascular / Spondylodiscitis	8192	8192	Neg	Pos	4

n.a., not available; [a]at the moment of diagnosis of chronic Q fever; [b]as measured with immunofluorescence assay (IFA, Focus Diagnostics); [c]at the moment of first blood sample for this study

IgG 1:131072 and a positive *C. burnetii* PCR in blood. Therapy with doxycycline and hydroxychloroquine was started and the patient underwent valvular replacement. PCR on valve tissue was positive for *C. burnetii* DNA. After 2 months therapy, PCR on blood was negative and remained so in the follow-up. The patient recovered well. Although he suffered from side effects (mainly photo-sensitivity), the antimicrobial therapy could be continued for 27 months, after which 3-monthly follow-up continued. By that time, the anti-phase I IgG had declined to 1:2048. After stop of antimicrobial therapy, the follow-up has been uneventful (13 months so far).

The IFN-γ/IL-2 ratio showed an initial increase, but from 5 months after start of treatment onward, the IFN-γ/IL-2 ratio declined to very low values (Figure 1B).

Case 3: a patient with *C. burnetii*-infected vascular prosthesis with failure of treatment

In the aftermath of the Q fever epidemic, a 64-years-old man presented with an acute aneurysm of the abdominal aorta. In the preceding weeks, he had back pain, fatigue, malaise, and weight loss. He underwent surgery with placement of a vascular prosthesis, and PCR on aorta tissue was positive for *C. burnetii*. Serology revealed elevated titers of anti-phase I and phase II IgG both 1:8192. PCR for *C. burnetii* DNA in blood was negative. He could not recall a preceding episode of fever or pneumonia. Doxycycline and hydroxychloroquine treatment was started. A transthoracic echocardiogram (TTE) showed thickening of the aortic valve. The patient refused to undergo TEE. He recovered well after the operation. However, anti-*C. burnetii* IgG titers did not decrease in the subsequent 2 years. PCR for *C. burnetii* DNA on blood, performed 3-monthly, remained negative. Moxifloxacin was added to the therapy after 23 months. FDG-PET/CT showed increased uptake at the vascular prosthesis, indicating a persistent infection. This patient showed an unsuccessful treatment of a chronic Q fever vascular infection. The IFN-γ/IL-2 ratio was relatively low from the start of therapy in this patient. The ratio showed an increase after the operation after which it remained mildly elevated, with no tendency to decline (Figure 1C). This was the result of neither decrease in IFN-γ production nor increase in IL-2 production over time.

An overview of the IFN-γ/Il-2 ratio in follow-up of 15 chronic Q fever patients

We studied the longitudinal IFN-γ/IL-2 ratio in fifteen chronic Q fever patients (Table 1). All were followed for at least 18 months during antibiotic treatment. In some cases, this included a period after completion of the treatment. For the purpose of this study, the data of all patients were analyzed according to start of antimicrobial therapy (t = 0), which was up to 35 months before inclusion. We

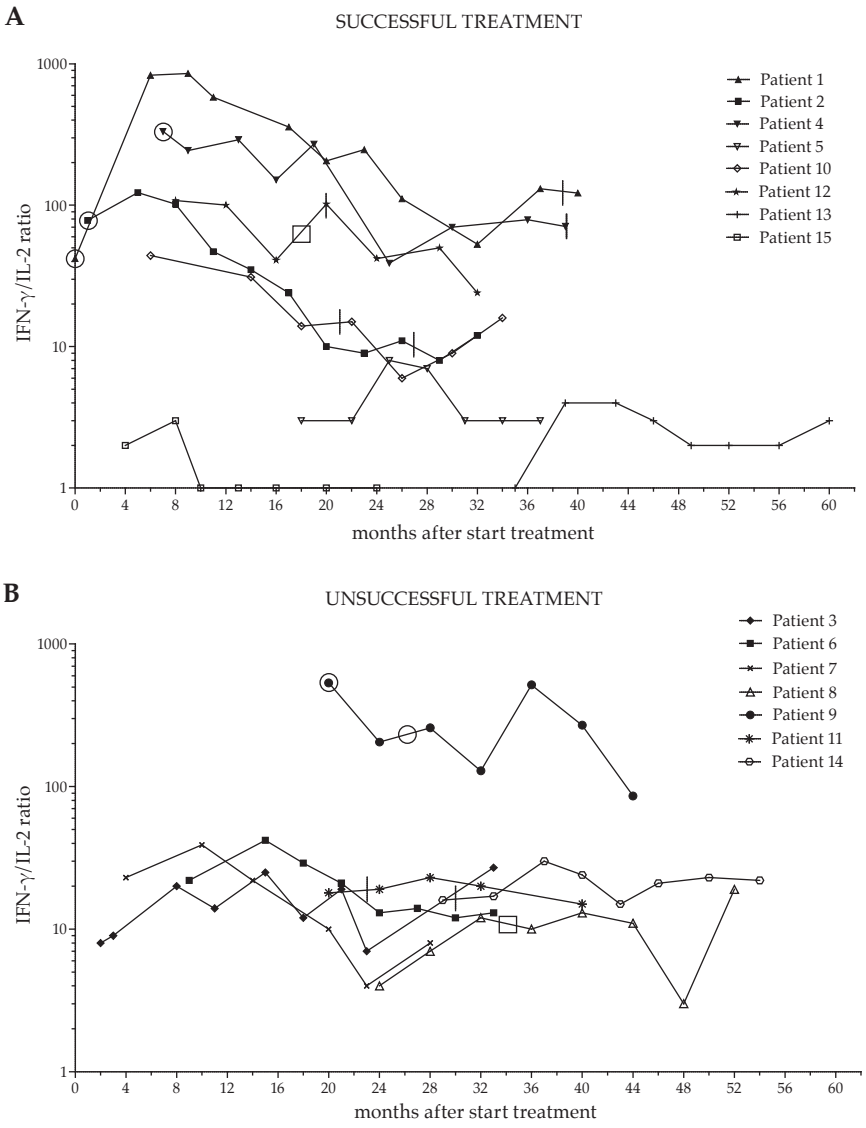

A
SUCCESSFUL TREATMENT
IFN-γ/IL-2 ratio
1000
100
10
1
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60
months after start treatment
Patient 1
Patient 2
Patient 4
Patient 5
Patient 10
Patient 12
Patient 13
Patient 15
B
UNSUCCESSFUL TREATMENT
IFN-γ/IL-2 ratio
1000
100
10
1
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60
months after start treatment
Patient 3
Patient 6
Patient 7
Patient 8
Patient 9
Patient 11
Patient 14
positive PCR for C. burnetii DNA on blood/serum
positive PCR for C. burnetii DNA on tissue
stop of anti-microbial therapy

Figure 2. Interferon-γ/IL-2 ratio in *C. burnetii*-stimulated whole blood of chronic Q fever patients during the study follow-up period, separately shown for (A) patients with successful and (B) patients with unsuccessful treatment. $t = 0$ is start of antibiotic treatment. Treatment was considered successful when 18 months of antibiotic treatment (or 24 months when a prosthesis remained *in situ*) was completed, and clinically recovery was observed, and a positive PCR for *C. burnetii* DNA on blood became persistently negative, and anti-phase I IgG showed a fourfold decrease or more (related to the maximum titer), and imaging techniques showed disappearance of any (vascular or valvular) infection focus. Circles indicate a positive PCR on blood/serum, squares indicate positive PCR on tissue. Vertical lines indicate stop of antimicrobial treatment.

divided the group in patients with successful treatment (n = 8) and those with unsuccessful treatment (n = 7; Figure 2). The latter group did not fulfill the success-criteria because of persistent PCR positivity for *C. burnetii* in blood (patient 9), less than a fourfold decrease in anti-phase I IgG titer (patient 3, 8, 11, 14), and/or persistent uptake on FDG-PET/CT (patient 3, 6, 7, 8, 14). As can be seen in Figure 2,

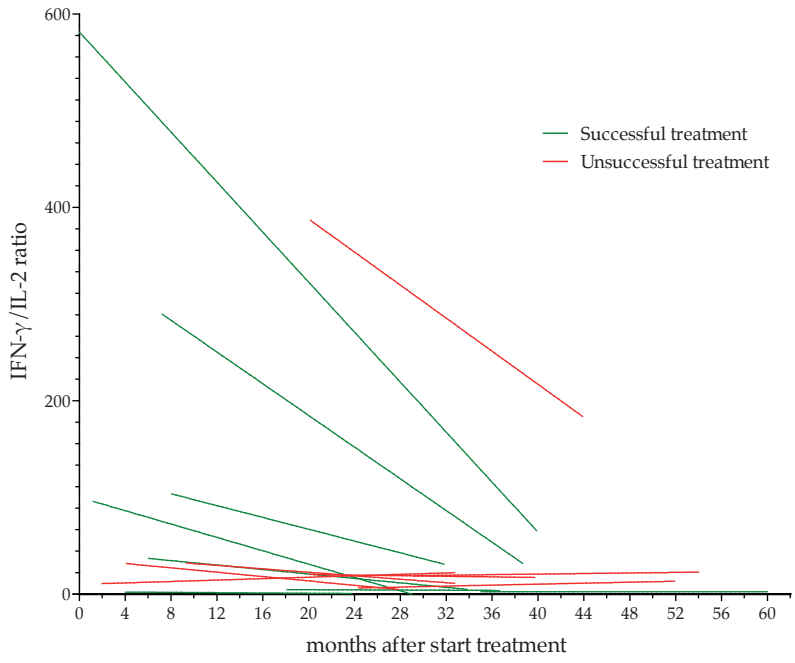


Figure 3. Non-linear regression to straight curves of the longitudinal IFN-γ/IL-2 ratios of chronic Q fever patients. Patients with successful treatment are shown in green, patients with unsuccessful treatment are shown in red. The median (±IQR) slope of the patients with successful treatment was −2.10 (−7.02 to −0.06), compared to −0.15 (−1.13 to 0.25) in patients with unsuccessful treatment (P = 0.19).

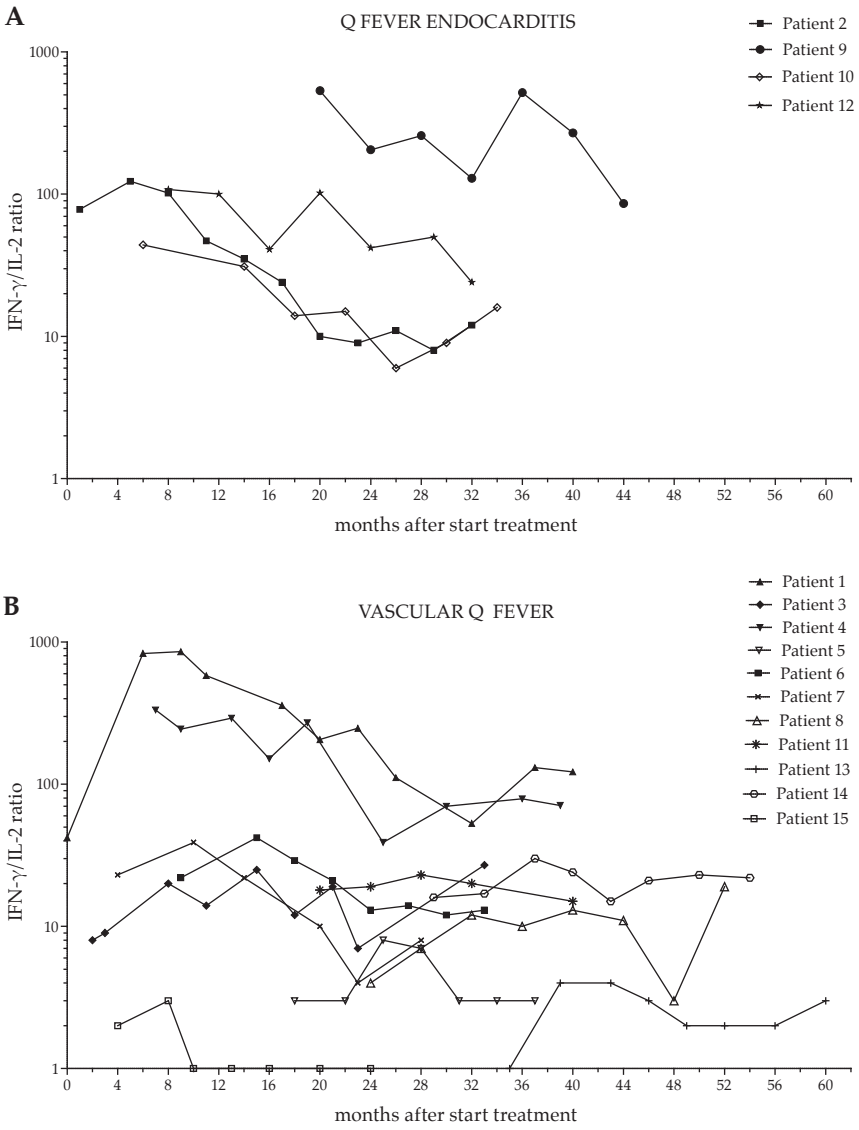


Figure 4. Follow-up IFN-γ/IL-2 ratio from chronic Q fever patients during the study follow-up period, separately shown for (A) patients with endocarditis and (B) patients with vascular (prosthesis) infection. $t = 0$ is start of antibiotic treatment.

the patients with successful treatment had higher maximum IFN-γ/IL-2 ratios than those with unsuccessful treatment. Moreover, the IFN-γ/IL-2 ratio of the patients with successful treatment showed a more pronounced decrease, each with an individual pattern, compared to the patients with unsuccessful treatment. We performed non-linear regression of the longitudinal IFN-γ/IL-2 ratios of each patient to a best-fit straight line (Figure 3). The median (±IQR) slope of the patients with successful treatment was −2.10 (–7.02 to −0.06), compared to −0.15 (–1.13 to 0.25) in patients with unsuccessful treatment (P = 0.19). Because the two main clinical manifestations of chronic Q fever may differ immunologically, the patients with endocarditis were also depicted separately from the patients with vascular (prosthesis) infection (Figure 4). Q fever endocarditis patients had overall higher IFN-γ/IL-2 ratios than the vascular Q fever patients.

Discussion

In the present study, we evaluated the usefulness of longitudinal measurements of cell-mediated immune responses against *C. burnetii* for treatment monitoring of chronic Q fever. We measured the *C. burnetii*-specific IFN-γ and IL-2 production in a whole-blood stimulation assay during a period of at least 18 months follow-up of proven chronic Q fever patients. We observed a trend in which the IFN-γ/IL-2 ratio declined when patients experienced a successful outcome of treatment. Patients in whom treatment failed, had overall lower IFN-γ/IL-2 ratios, which did not significantly decrease.

The IFN-γ/IL-2 ratio reflects the type of effector and memory $CD4^+$ T-cell response (Sallusto et al., 1999). Memory T lymphocytes can broadly be defined as two distinct populations of effector memory T-cells and central memory T-cells. IFN-γ is predominately produced by effector T-cells and effector memory T-cells, while IL-2 is predominately produced by central memory T-cells. It is postulated that high IFN-γ/IL-2 ratio indicates predominance of effector T-cells and effector memory T-cells, resulting from ongoing immunological stimulation by a persistent infection.

The relevance of measuring IFN-γ/IL-2 production profiles, as diagnostic correlate of memory T-cell responses, has been studied outside the field of Q fever, specifically in a number of viral infections (Younes et al., 2003; Semmo et al., 2005), and in *Mycobacterium tuberculosis* infection (Sargentini et al., 2009; Biselli et al., 2010; Casey et al., 2010; Sester et al., 2011; Essone et al., 2014). These studies, as summarized by Lalvani and Millington (2008), show that in acute and chronic infections with a high antigen concentration, e.g., in chronic progressive HCV infection (Semmo et al., 2005) or untreated tuberculosis (Millington et al., 2007),

$CD4^+$ T-cells predominately secrete IFN-γ. In infections with persistently low antigen concentrations, e.g., latent asymptomatic cytomegalovirus infection, $CD4^+$ T-cells secreting IFN-γ only, IFN-γ/IL-2, or IL-2 only are detected (Harari et al., 2005). In cleared infections, IL-2 secreting $CD4^+$ T-cells predominate (Harari et al., 2004, 2005; Correa et al., 2007; Millington et al., 2007). Our finding that the IFN-γ/IL-2 ratio declines during successful treatment of chronic Q fever, assuming a decrease in antigen load, is in accordance with these studies.

The group of patients that was included in our study was inevitably heterogeneous with regard to morbidity, infection status, treatment course and treatment response. Likewise, the inter-individual variation in IFN-γ and IL-2 responses was large. We considered it therefore not feasible to combine individual results for a grouped analysis, and chose to describe patients separately. Three patients were described in more detail, because they were followed (almost) from the start of antibiotic treatment. Strikingly, in two of these patients (patients 1 and 2), who seemed to respond well to treatment, the IFN-γ production and the IFN-γ/IL-2 ratio initially increased, but decreased thereafter. It is tempting to speculate that this initial increase reflects an adequate immune response. A similar initial increase of the specific IFN-γ response is seen in patients during treatment for tuberculosis (Sahiratmadja et al., 2007). The patient that still showed signs of infection after more than 2 years of antibiotic treatment (patient 3), had markedly high IL-2 secretion and a low IFN-γ/IL-2 ratio from the start, which fluctuated in the follow-up but did not decrease. This might suggest bacterial persistence with low antigen concentrations; an assumption that is supported by the notion that *C. burnetii* DNA was not detectable in blood, even before start of antimicrobial therapy. The results of the total group of patients with unsuccessful treatment show the same pattern: overall lower IFN-γ/IL-2 ratio from the start of follow-up in this study, with no *C. burnetii* DNA detectable in blood. An exception to this rule is patient 9, who failed on antibiotic treatment by having *C. burnetii* DNA detectable in blood after 20 months of antimicrobial therapy. The lower IFN-γ/IL-2 ratio we observed in patients with endovascular infections compared to endocarditis patients suggests that these manifestations of chronic Q fever differ with respect to antigen concentrations; vascular infections might be a more low-grade infection than endocarditis.

Our study has some limitations. First of all, we studied a relatively small number of patients. Longer follow-up with additional time points after completion of treatment need to be incorporated into future studies to evaluate success of treatment. Moreover, the method we used, *in vitro* measurement of IFN-γ and IL-2 production, does not clearly differentiate whether these cytokines are produced by effector T-cells (producing only IFN-γ) or effector memory T-cells (producing IFN-γ and IL-2) or central memory T-cells (producing predominantly IL-2). The

ratio IFN-γ/IL-2 merely reflects the overall result and might be influenced by the total number of circulating T-cells and their viability *in vitro*. To increase our insight in the matter, detection of cytokine production on single-cell level, e.g., by flow cytometry with intra-cellular cytokine staining (Harari et al., 2005), would be a valuable addition in future research.

The central position of serology in Q fever diagnostic is undisputable, and serology has hitherto been the most widely used immunological measurement for *C. burnetii* infection. It continues to be extensively validated in Q fever research (Frankel et al., 2011; van der Hoek et al., 2011; Edouard et al., 2013; Herremans et al., 2013). Nevertheless, the immunological importance of measuring antibodies in response to this intracellular bacterial infection is questionable. Specifically, the use of antibody titers to monitor the effect of antibiotic treatment on *C. burnetii* infection and disease needs further research. The definition of serological cure as anti-phase I IgG below 1:800 (or 1:1024 when a commercial IFA is used) is based on expert opinion (Million et al., 2010). The definition of absence of a fourfold decrease in antibody titers as a poor prognostic factor, is based on a smallsized retrospective study of Q fever endocarditis (Million et al., 2010), and is not yet confirmed by other studies. In the light of this limited evidence of serology as a biomarker, it would be valuable to also focus on the more relevant cell-mediated immune response. Especially when the decision to continue or stop treatment has to be made in an individual patient, the availability of other relevant biomarkers may be of help. Our study shows a promising role for the IFN-γ/IL-2 production profile, although the large variation in IFN-γ/IL-2 ratio between patients in this study makes it difficult to formulate general recommendations for the application of these biomarkers at the current time.

In conclusion, existing clinical, imaging and microbiological parameters to monitor the response to treatment have several limitations. We propose that the IFN-γ/IL-2 production profile can be used as an additional immunological biomarker for treatment monitoring of chronic Q fever.

Acknowledgments

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8

Diagnosis of *Coxiella burnetii* infection: comparison of a whole blood interferon-gamma production assay and a *Coxiella* ELISPOT

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Abstract

Diagnosis of ongoing or past infection with *Coxiella burnetii*, the causative agent of Q fever, relies heavily on serology: the measurement of *C. burnetii*-specific antibodies, reflecting the host's humoral immune response. However, cell-mediated immune responses play an important, probably even more relevant, role in infections caused by the intracellular *C. burnetii* bacterium. Recent studies have investigated interferon-gamma (IFN-γ) based assays, including a whole-blood IFN-γ production assay and a *Coxiella* enzyme-linked immunospot (*Coxiella* ELISPOT), as potential diagnostic tools for Q fever diagnosis. Both are in-house developed assays using stimulating antigens of different origin. The main objective of this study was to compare the test performance of the IFN-γ production assay and the *Coxiella* ELISPOT for detecting a cellular immune response to *C. burnetii* in Q fever patients, and to assess the correlation between both assays. To that end, both tests were performed in a well-defined patient group of chronic Q fever patients (n = 16) and a group of healthy seronegative individuals (n = 17). Among patients, both the Coxiella ELISPOT and the IFN-γ production assay detected positive response in 14/16. Among controls, none were positive in the Coxiella ELISPOT, whereas the IFN-γ production assay detected positive results in 1/17 and 3/17, when using Henzerling and Nine Mile as stimulating antigens, respectively. These results suggest the *Coxiella* ELISPOT has a somewhat higher specificity than the IFN-γ production assay when Nine Mile is used as antigen stimulus. The assays showed moderate correlation: the Spearman correlation coefficient *r* ranged between 0.37–0.60, depending on the antigens used. Further investigation of the diagnostic potential for *C. burnetii* infection of both assays is warranted.

Introduction

Q fever is a zoonotic disease that occurs worldwide and is caused by the gram-negative, intracellular bacterium *Coxiella burnetii*. The Netherlands experienced a major Q fever outbreak between 2007 and 2010, with over 4,000 reported human infections [1]. Acute Q fever mostly presents as a flu-like illness, pneumonia or hepatitis, but initial infections can be asymptomatic in more than 50% of cases [2]. Chronic Q fever is a rare but serious complication that occurs in approximately 5% of all patients following the acute infection. This persisting infection typically presents with endocarditis or vascular infection, and has significant mortality rates, especially in case of diagnostic and therapeutic delay [3].

Evaluating chronic *C. burnetii* infection is challenging. Measurement of serum antibodies against *C. burnetii* is currently the 'gold standard' for Q fever diagnosis [3]. Diagnosis of chronic Q fever relies heavily on detection of high IgG antibody titers against phase 1 *C. burnetii*. This serological criterion is combined with PCR for *C. burnetii* DNA on blood or tissue (if available) and clinical assessment of any nidus of chronic infection [4,5].

Both humoral and cell-mediated immune responses are involved in the host's immunity against the intracellular *C. burnetii* bacterium [6–8]. Therefore, it makes sense to explore the value of complementing conventional serology with an assessment of host specific cell-mediated immune responses to detect a chronic infection with *C. burnetii*. To this end, new immunological blood tests have been developed that are based on cellular immunity, measuring T-cell derived interferon-γ (IFN-γ) production in response to stimulation with *C. burnetii*. The first, the whole-blood IFN-γ production assay, was extensively investigated during a Q fever vaccination campaign and for the diagnosis of chronic Q fever [9–11]. The second, the *Coxiella* enzyme-linked immunospot (*Coxiella* ELISPOT), was explored in a small series of patients with past or chronic Q fever [12]. The IFN-γ production assay measures the amount of *C. burnetii* specific IFN-γ production, while the *Coxiella* ELISPOT measures the number of *C. burnetii* specific IFN-γ producing cells. These IFN-γ based assays are not yet routinely used for Q fever diagnosis. Both are in-house assays, using different stimulating antigens. The IFN-γ production assay uses in-house cultured Nine Mile phase 1 and the Q-vax vaccine containing Henzerling phase 1, while the *Coxiella* ELISPOT uses commercially available Nine Mile phase 1 and phase 2 antigens.

In the aftermath of the Dutch Q fever outbreak, we had the opportunity to use both IFN-γ based tests in parallel in a group of well-defined chronic Q fever patients. Volunteers with no history of Q fever and with negative Q fever serology served as a control group.

The purpose of this study was to compare the test performance (sensitivity and specificity) of the IFN-γ production assay and the *Coxiella* ELISPOT for detecting a cellular immune response to *C. burnetii* in Q fever patients and to determine the correlation between the assays.

Materials and Methods

Patients and control subjects

Chronic Q fever patients (n = 16) were recruited from the outpatient clinics of the participating hospitals. All fulfilled the criteria for probable (n = 4) or proven (n = 12, of which 3 Q fever endocarditis and 9 Q fever vascular infection) chronic Q fever according to the Dutch consensus statements on chronic Q fever [4]. Fourteen of the 16 patients were on long-term antibiotic treatment at inclusion. The median duration of antibiotic treatment at the time of blood collection was 21 months (range 0–59 months). The control individuals (n = 17) were similar with respect gender, somewhat younger, but had no history of Q fever and had negative Q fever serology as measured by immunofluorescence assay (Focus Diagnostics, Cypress, CA, USA) (Table 1). Informed consent was obtained from all subjects before blood donation and the study was approved by the local ethics committee (CMO regio Arnhem-Nijmegen).

IFN-γ production assay

The IFN-γ production assay was performed as previously described [9]. In short, heparinized whole blood was aliquoted into four separate 1.5 mL tubes at 0.5 mL per tube. The tubes were inoculated with either 10 μg/mL PHA (Sigma, St. Louis, MO), 100 ng/ml Q vax vaccine (see below), 10^7/mL heat-inactivated *C. burnetii* Nine Mile phase I (see below), or nothing. The tubes were incubated in-vitro for 24 hours at 37°C and 5% CO_2. The IFN-γ production was measured in the supernatant by ELISA (Pelikine compact, Sanquin, Amsterdam). Net IFN-response was expressed as the concentration of IFN-γ in the stimulated sample minus that in the unstimulated sample.

The Q-vax vaccine (CSL Biotherapies, Vic., Australia), contains formalin-inactivated whole cell phase 1 Henzerling strain.

Heat-inactivated phase 1 *C. burnetii* Nine Mile (NMI, RSA493) was kindly provided by H.I. Roest (Central Veterinary Institute, Lelystad, the Netherlands). Details about culture and preparation of this stimulating antigen are described elsewhere [9].

Table 1 Characteristics of chronic Q fever patients and control individuals

	Patients (n = 16)	Controls (n = 17)
Age in yrs, median (range)	68 (31–80)	46 (25–64)
Males, number (%)	13 (81)	14 (82)
IgG anti-phase I titer[a], median (range)	2048 (128–32768)	<16 (n.a.)
IgG anti-phase II titer[a], median (range)	3072 (256–32768)	<16 (n.a.)
Duration of antibiotic treatment in months[a], median (range)	21 (0–59)	n.a.

n.a., not applicable.
[a] At the time of bloodsampling.

Coxiella ELISPOT

The *Coxiella* ELISPOT was performed on peripheral blood mononuclear cells (PBMCs) isolated from heparinized blood and stimulated for 16–20 hours, as described before [12].

In precoated wells of PVDF strip plates (ELISpotpro; Mabtech) 100 μL of mononuclear cells were seeded at a density of 250,000 cells per well, and incubated with 50 μL of antigens, PHA (2.5 μg/ mL) or nothing. As stimulating antigens, commercially available formalin-inactivated phase 1 and phase 2 Nine Mile (NMI and NMII, Virion-Serion Immunodiagnostica GmbH, Würzburg, Germany) were used. After incubation, the resulting number of spots, representing the number of individual T-cells producing IFN-γ following stimulation with *C. burnetii* antigens, were detected and enumerated using an ELISpot reader (Auto Immun Diagnostika GmbH, Strassberg, Germany).

Statistics

Statistical analysis was performed using GraphPad Prism 5. Median (6 interquartile range) IFN-γ production and number of spots were compared between groups using Mann-Whitney *U*-tests. Receiver Operating Characteristics (ROC) curves analysis were used to derive a cutoff for positivity of either assay. The cutoff was determined from the ROC curve by choosing the value that yielded empirical optimal sensitivity and specificity.

Correlation was reported by calculating the Spearman's *r* with 95% Confidence Interval.

Results and Discussion

The amount of IFN-γ as measured in the IFN-γ production assay and the spot count obtained in the *Coxiella* ELISPOT were evaluated. The individual values are shown in Figure 1 for patients and control subjects separately. The median IFN-γ response to the Henzerling antigen in the patients was 440 pg/mL (IQR 62–612 pg/mL), whereas in control subjects, this was 0 pg/mL (IQR 0–0 pg/mL). The median IFN-γ response to the NMI antigen in the patients was 3238 pg/mL (IQR 534–6564 pg/mL), whereas in control subjects, this was 126 pg/mL (IQR 14–250 pg/mL). The median ELISPOT spot count after NMI stimulation was 44 (IQR 15–94) in patients and 0 (IQR 0–0) in controls, and after NMII stimulation 71 (IQR 23–100) in patients and 0 (IQR 0–0) in controls. In both assays, the differences between patients and control subjects were significant for each stimulating antigen. All of the samples tested responded to PHA mitogen.

To establish a cutoff for a positive response in both assays for each of the antigens, ROC curves were constructed (Figure 2). Cutoffs were derived from ROC curve analysis to yield empirical optimal sensitivity and specificity. For the IFN-γ production assay, this resulted in a cutoff of 45 pg/mL or 365 pg/mL when using

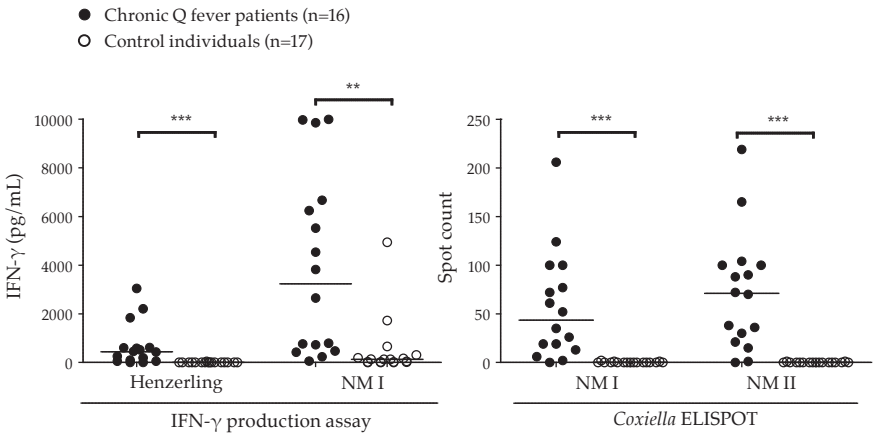

Figure 1. Results of the IFN-γ production assay and *Coxiella* ELISPOT in chronic Q fever patients and control subjects. *In vitro* IFN-γ production by whole blood in response to Henzerling and Nine Mile phase 1 antigens was measured in the IFN-γ production assay. The number of IFN-γ positive cells in response to Nine Mile phase 1 and Nine Mile phase 2 antigens was measured in the *Coxiella* ELISPOT. Individual values of patients and controls are shown separately, and the lines indicate the medians. Patients and controls were compared using the Mann-Whitney *U*-test.
****P*,0.001, ***P*,0.01. Abbreviations: NMI, Nine Mile phase 1; NMII, Nine Mile phase 2; IFN-γ, interferon-gamma.

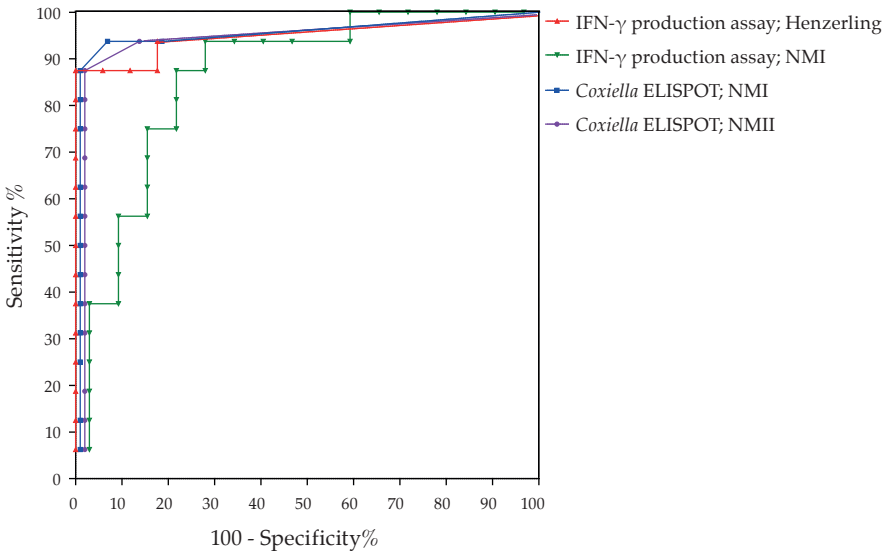

Figure 2. ROC curves of the IFN-γ production assay and the *Coxiella* ELISPOT. ROC curves are shown for each assay with each stimulating antigen separately. Abbreviations: NMI, Nine Mile phase 1; NMII, Nine Mile phase 2; IFN-γ, interferon-gamma.

the Henzerling or the NMI as stimulating antigen respectively. For the *Coxiella* ELISPOT, this resulted in a cutoff of 5 spots to both NMI and NMII. Given these cutoffs, the IFN-γ production assay detected positive response in 14/16 and the *Coxiella* ELISPOT was positive in 14/16 among the patients. Among the controls, the IFN-γ production assay detected positive response in 1/17 and 3/ 17, when using Henzerling and Nine Mile as stimulating antigens respectively, whereas none were positive in the *Coxiella* ELISPOT. These results suggest that the assays have comparable sensitivity in this population, but the *Coxiella* ELISPOT has a higher specificity than the IFN-γ production assay with Nine Mile as an antigen stimulus. This might be due to different antigens being exposed after heat-inactivation of the NMI strain as compared to the antigens present on the formalin-inactivated bacteria.

Furthermore, it is very well possible that the IFN-gamma production assay and the *Coxiella* ELISPOT assay do not measure the same features of the immune response. Although both tests measure *Coxiella* specific IFN-gamma production, the *Coxiella* ELISPOT assay singles out and quantifies the T lymphocyte compartment, whereas whole blood stimulation also includes blood IFN-gamma producing NK cells, reflecting also the innate part of the immune response.

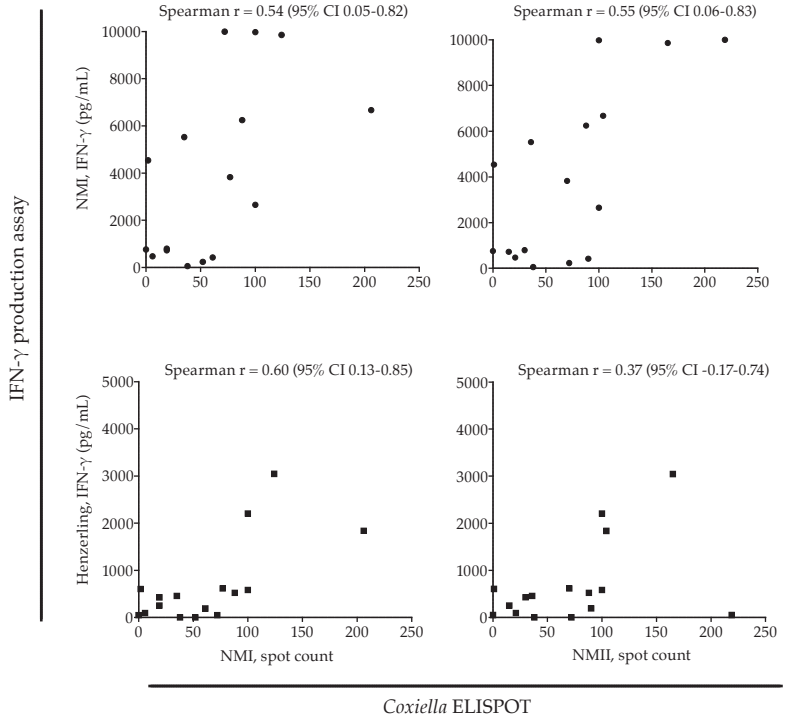

Figure 3. Correlation between the amount of *C. burnetii*-specific IFN-γ production and the number of IFN-γ positive cells. The individual values of chronic Q fever patients (n = 16) were used to determine the correlation between the IFN-γ production as measured with the IFN-γ production assay, and the number of IFN-γ positive cells as measured with the *Coxiella* ELISPOT. Each graph shows the correlation between the resulting values of the two assays, with either one of the stimulating antigens. On the Y-axes, the IFN-γ production is shown after stimulation with NMI (upper graphs) and Henzerling antigens (lower graphs). On the X-axes, the spot count is shown after stimulation with NMI (left graphs) and NMII (right graphs) antigens. The Spearman's correlation coefficient *r* (95% Confidence Interval) is given for each comparison. Abbreviations: NMI, Nine Mile phase 1; NMII, Nine Mile phase 2; IFN-γ, interferon-gamma.

However, true positives in the control group cannot be excluded which in turn may underestimate specificity. A seroprevalence study in the Q fever epidemic area among patients with a valvular risk factor for chronic Q fever found 20.4% of the people to be seropositive for *C. burnetii* [13]. As the control subjects lived close to or in the epidemic area, although not having a Q fever history or detectable anti-*C. burnetii* antibodies, it is not unthinkable that any of the control subject could have a past exposure to *C. burnetii* that was not picked up by serology. Of note, two of the three control subjects that produced substantial amounts of IFN-γ

after NMI antigen stimulation, also showed minimal response below the cutoff in the *Coxiella* ELISPOT.

In the patient samples, we determined the correlation between the amount of IFN-γ produced in the IFN-γ production assay and the number of IFN-γ-producing cells in the *Coxiella* ELISPOT (Figure 3). The correlation between the values obtained in the two assays, each employing two stimulating antigens, was determined separately, resulting in a total of four comparisons. The Spearman correlation coefficient *r* ranged between 0.37–0.60, indicating a moderate to strong correlation. The discrepancies between both types of assays can be explained by the different origins and concentrations of stimulating antigens, the different phase variation, or, as mentioned previously, the method of inactivation of the antigen stimuli. Moreover, it may well be true that the amount of IFN-γ released is not directly related to the number of IFN-γ positive cells. IFN-γ based assays are used for detection of immunity to *Mycobacterium tuberculosis* and commercial kits are available for the two types of assays, e.g. the QuantiFERON-TB and the T Spot TB [14]. These assays have been reported to be at least as accurate as the tuberculin skin test to detect exposure to *M. tuberculosis*. The correlation between the two types of IFN-γ based assays for tuberculosis as previously reported in the literature, are better ($r = 0.69$ and $r = 0.80$) than what we observed evaluating these diagnostic platforms in *C. burnetii* infection [15,16].

In conclusion, the two IFN-γ based assays had a similarly high sensitivity for detecting *C. burnetii* infection and the correlation between both test was moderate. The IFN-γ production assay in whole blood has the practical advantage of relative technical simplicity over the more laboriously intensive *Coxiella* ELISPOT. The *Coxiella* ELISPOT, however, seemed to be more specific with Nine Mile as an antigen stimulus. These observations, and previous studies of these IFN-γ based assays in *C. burnetii* infection, show the potential of measuring cell-mediated immune response in Q fever and warrant further investigation of these assays in larger cohorts of Q fever patients.

Acknowledgments

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9

Early cytokine and antibody responses against *Coxiella burnetii* in aerosol infection of BALB/c mice

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Abstract

Coxiella burnetii, a Gram-negative intracellular bacterium, can give rise to Q fever in humans and is transmitted mainly by inhalation of infected aerosols from animal reservoirs. Serology is commonly used to diagnose Q fever, but the early cellular immune response—i.e., *C. burnetii*–specific interferon γ (IFN-γ) production in response to antigen challenge—might be an additional diagnostic. Detection of IFN-γ responses has been used to identify past and chronic Q fever infections, but the IFN-γ response in acute Q fever has not been described. By challenging immunocompetent BALB/c mice with aerosols containing phase I *C. burnetii*, the timing and extent of IFN-γ recall responses were evaluated in an acute *C. burnetii* infection. Other cytokines were also measured in an effort to identify other potential diagnostic markers. The data show that after initial expansion of bacteria first in lungs and then in other tissues, the infection was cleared from day 10 onwards as reflected by the decreasing number of bacteria. The antigen-induced IFN-γ production by splenocytes coincided with emergence of IgM phase II antibodies at day 10 postinfection and preceded appearance of IgG antibodies. This was accompanied by the production of proinflammatory cytokines including interleukin (IL) 6, keratinocyte-derived cytokine, and IFN-γ–induced protein 10, followed by monocyte chemotactic protein 1, but not by IL-1β and tumor necrosis factor α, and only very low production of the anti-inflammatory cytokine IL-10. These data suggest that analysis of antigen-specific IFN-γ responses could be a useful tool for diagnosis of acute Q fever. Moreover, the current model of *C. burnetii* infection could be used to give new insights into immunological factors that predispose to development of persistent infection.

Introduction

Infection with *Coxiella burnetii*, a Gram-negative intracellular bacterium, causes Q fever in humans. The common route of infection is through inhalation of *C. burnetii*–infected aerosols spread from animals, usually sheep or goats (McQuiston and Childs, 2002). Acute Q fever presents as a flu-like illness but can be asymptomatic in over 50% of infections. A minority of cases presents as pneumonia or hepatitis (Parker et al., 2006). Generally, acute Q fever is self-limiting, yet early recognition and antibiotic treatment may shorten duration (Benenson and Tigertt, 1956). In some cases, however, *C. burnetii* infection leads to a chronic infection (chronic Q fever), mostly Q fever endocarditis or vascular infection (Million et al., 2010). These conditions are life-threatening if left untreated. Prevention of evolution from acute to chronic Q fever, by prolonged antibiotic treatment following initial infection, is suggested for risk groups, but the value of this intervention is debated (Limonard et al., 2011; Million et al., 2013).

In the initial phase of the infection, cytokines and chemokines produced by monocytes and macrophages are central to recruit and activate other immune cells, promote pathogen disposal, and develop adaptive immunity. Cell-mediated adaptive immune responses are essential for control of acute *C. burnetii* infection, probably even more important than B-cell responses (Andoh et al., 2007; Read et al., 2010; Sidwell et al., 1964). *C. burnetii*–specific T cells produce interferon-γ (IFN-γ) and activate monocytes/macrophages to produce inflammatory cytokines and control intracellular *C. burnetii* growth (Brennan et al., 2004; Ghigo et al., 2002).

Currently, detection of acute Q fever infection in humans mainly relies on measurement of *C. burnetii*–specific serum antibodies. Measurements of T-cell immune responses might be of additional value in acute Q fever but, so far, have not been investigated in this context. To obtain data on early adaptive immune responses, human studies are of limited value, since patients are identified fairly late in the course of overt clinical disease. Animal models that mimic human acute Q fever can be used instead.

Animal models for Q fever usually include guinea pigs or mice (Maurin and Raoult, 1999). In mice, as in humans, *C. burnetii* infection can cause disease, with different mouse strains showing divergent vulnerability for infection, with mortality only in the most sensitive strains (Scott et al., 1987). The incubation time till development of symptoms depends on the inoculation dose (Benenson and Tigertt, 1956), the route of infection, and the phase of *C. burnetii*. The virulent form is the so-called phase I *C. burnetii* that possesses a full-length lipopolysaccharide (LPS) and is isolated from infected humans or animals (Russell-Lodrigue et al., 2009). Phase II, obtained after several passages of phase I organisms in vitro, displays a truncated LPS molecule lacking the terminal O-antigen sugars (Amano

et al., 1987) and does not lead to disease even when administered in high inocula in experimental animals (Andoh et al., 2005, 2007; Moos and Hackstadt, 1987). The route of infection is of importance with a shorter incubation time in animals infected intraperitoneally as compared to the natural route of respiratory infection (Tigertt et al., 1961). Clearly, aerosol infection resembles most closely the natural route of infection in humans and should be preferred for studying the disease (Stein et al., 2005).

The main purpose of this study was to investigate the development of cellular immunity—i.e., *C. burnetii*–specific IFN-γ production in response to antigen challenge—and to compare this with the timing of IgM and IgG antibody responses against phase I and II bacteria. In addition, we investigated the specific production of early inflammatory mediators—a panel of monocyte-/macrophage-derived cytokines and chemokines—in an effort to identify other potential diagnostic markers. To facilitate analysis and mimic the mode of transmission for human acute Q fever most closely, we used a mouse model of aerosol infection with phase I *C. burnetii* in immunocompetent BALB/c mice.

Materials and methods

Animals

A total of 50 male BALB/c mice, 9 weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). This mouse strain is known to be intermediately sensitive to infection with *C. burnetii* (Scott et al., 1987; Stein et al., 2005). Mice were housed in a Tecniplast Isocage system (Tecniplast, Exton, PA, USA) in an ABSL3 facility and given food and water ad libitum. The animal experiments were performed according to an animal protocol approved by the CDC Institutional Animal Care and Use Committee.

Bacteria

The strain used for this study was *C. burnetii* Nine Mile (NM) phase I (RSA493). This reference strain, isolated from a tick in 1935 (Maurin and Raoult, 1999), can cause Q fever in humans (Benenson and Tigertt, 1956) and grows well in mouse models (Russell-Lodrigue et al., 2009). It was grown in chicken eggs and purified by sucrose gradient centrifugation (Miller and Thompson, 2002). Stockswere kept frozen at–80 °C in sucrose phosphate glutamate buffer until use.

Mouse infections

On day 0, 40 mice were inoculated using the Biaera aerosol management platform (AeroMP; Biaera Technologies, Hagerstown, MD, USA). Ten milliliters of phosphate-buffered saline (PBS) containing NM phase I bacteria (at 10^8 organisms/mL) was

placed in a nebulizer, and the aerosolized bacteria were introduced into the chamber containing the 40 mice for a 10-minute exposure period. Sixty liters of air from the chamber was sampled in an impinger containing 10-mL PBS. Quantitative PCR detected 1.68×10^7 C. burnetii organisms in the impinger, suggesting that the air in the chamber contained 280 organisms permilliliter of air. Based on a tidal volume of 0.15mL and a respiratory rate of 163/min for mice, it is estimated that each mouse inhaled 6.8 × 104 C. burnetii organisms. Ten mice served as a negative control group and were left uninfected. The infected and uninfected mice were maintained in separate high efficiency particulate air-filtered isolator cages.

On day 1, 3, 7, 10, and 14, groups of 8 infected and 2 uninfectedmice were euthanized by exsanguination under isoflurane anesthesia, after which the euthanasia was verified by cervical dislocation. Blood was harvested by cardiac puncture and collected in heparinized tubes, and blood from pairs of mice was pooled. Lungs, spleen, and liver were aseptically removed. Spleens were weighed before further processing.

Quantitative PCR

For analysis of the quantity of *C. burnetii* DNA in blood and tissue, blood and spleens from the 8 infected and 2 uninfected mice at each time point were pooled into 5 pairs. Spleens were homogenized into single cell suspensions by grinding the tissues between frosted ends of ground glass slides before pooling. For liver and lung, the organs from each mouse were tested independently. To quantify the *C. burnetii*, total genomic DNA was extracted from 100-μL blood, lung/liver tissue, or spleen cell suspensions using the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. On all these samples, quantitative PCR for *IS1111a* was performed as described (Kersh et al., 2010).

Serology

Serum titers of IgM and IgG antibodies against phase I and II *C. burnetii* were determined by indirect immunofluorescence antibody test (IFA). Plasma was obtained from heparinized blood through centrifugation at 1200 × g. Slides coated with either NM phase I (RSA 493) or NM phase II (RSA 439) strains were incubated with titrations of plasma samples. After washing, they were treated with fluorescein isothiocyanate–conjugated goat anti-mouse antibody, and binding was visualized using a fluorescence microscope. The greatest dilution of plasma that resulted in unambiguous antibody binding is reported as titer.

Splenocyte stimulation

Splenocytes were isolated by homogenizing the spleens by grinding the tissue between the frosted ends of a pair of ground glass slides, creating single-cell

suspensions in sterile PBS. Splenocytes from pairs of mice were pooled. After centrifugation at 300 × g for 10 minutes at 20 °C, red blood cells were lysed by osmotic shock followed by resuspension in PBS. After passage through a 100-μm cell strainer to filter debris, cells were centrifuged again at 300 × g for 10 minutes at 20 °C and resuspended in RPMI culture media supplemented as described. Cells were plated at 3×10^6 cells/well in a 24-well plate, in a final volume of 1 mL per well.

Splenocyteswere stimulated with medium alone (negative control), the mitogen concanavalin A (conA) (2.5 μg/mL), or heat-killed (60 minutes, 80 °C) (Enright et al., 1957) phase I NM at either 1×10^6/mL or 1×10^7/mL. After 48 h incubation at 37 °C and 5% CO_2, 400 μL of splenocyte supernatant was collected from each well. Supernatantswere stored at–80 °C until cytokines were measured.

Cytokine analysis

Supernatant samples were gamma-irradiated (2×10^6 rads) before handling. Cytokine concentrations—including mouse interleukin (IL) 1β, IL-6, tumor necrosis factor (TNF) α, IL-10, keratinocyte-derived cytokine (KC), monocyte chemotactic protein (MCP) 1, IFN-γ–induced protein (IP) 10, and IFN-γ—were measured using a Luminex bead– based multiplex assay (R and D Systems, Minneapolis, MN, USA), in accordance with the manufacturers' instructions. Samples were analyzed using a Bio-Plex Luminex 100 (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data are expressed as mean ± SD (for weight and genome copies) or median ± IQR (for cytokine data). Differences between uninfected and infected mice at different time points after infection were tested using analysis of variance (ANOVA) test or Kruskal–Wallis test, as appropriate. GraphPad Prism 5.0 software (GraphPad) was used. A difference was considered significant if the *P* value was ≤0.05.

Results

C. burnetii infection in mice

All infected mice showed some signs of lethargy and ruffled fur for 2–7 days, with onset between day 7 and day 14 postinfection. Infected mice developed splenomegaly from day 3 onwards (Fig. 1). None of the mice died before being sacrificed.

Detection of *C. burnetii* DNA

The bacterial DNA copy numbers in the lungs of infected mice increased from day 1 to day 7 and declined thereafter. In plasma, amplification products were obtained at day 3 (only in 1 of 4 pairs of mice) and reached maximum at day 7 (all pairs of

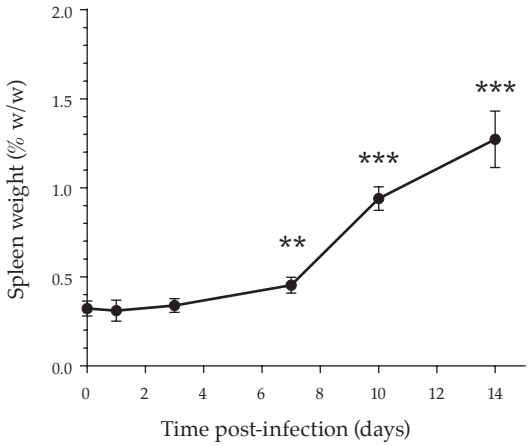

Figure 1. Spleen weight after aerosol infection with *C. burnetii* in immunocompetent BALB/c mice. Spleen-to-body weight (mean ± SD) is shown for 10 uninfected mice (t = 0) and 8 infected mice per time point postinfection. ANOVA test followed by Dunn's multiple comparison test was used to compare infected mice at different time points with uninfected mice. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

mice). In liver and spleen, *C. burnetii* DNA was detected at day 7 and reached maximum at day 10 afterwhich it declined (Fig. 2). At day 14, of the tissues examined, spleens contained the highest load of *C. burnetii* DNA. No bacterial DNA was detected in plasma and tissue of uninfected mice.

Serological response

Serological responses, asmeasured by IFA, are shown in Fig. 3. A positive response in infected mice was first detectable at day 10, with low titers (ranging from 1:32 to 1:64) for IgMphase II. These titers increased to 1:256 to 1:2048 at day 14. IgG phase II and IgMphase Iwere also positive in all infectedmice (range 1:256 to 1:512 and range 1:64 to 1:256, respectively). At day 14, all infected mice had developed antibodies against NMphase I and II *C. burnetii*, and all control mice remained seronegative. IgG against phase I was only lowpositive in 2 of 4 mouse pairs (maximum 1:32) at day 14.

Cytokine production

Cytokinesweremeasured in supernatants of splenocytes stimulated for 48 h in vitro. Splenocytes of infected mice produced substantial amounts of cytokines, with different stimulus-dependent, time postinfection patterns. The pattern of antigen-induced IFN-γ production, reflecting a specific cell-mediated immune response, was of special interest (Fig. 4). conA-induced IFN-γ production was

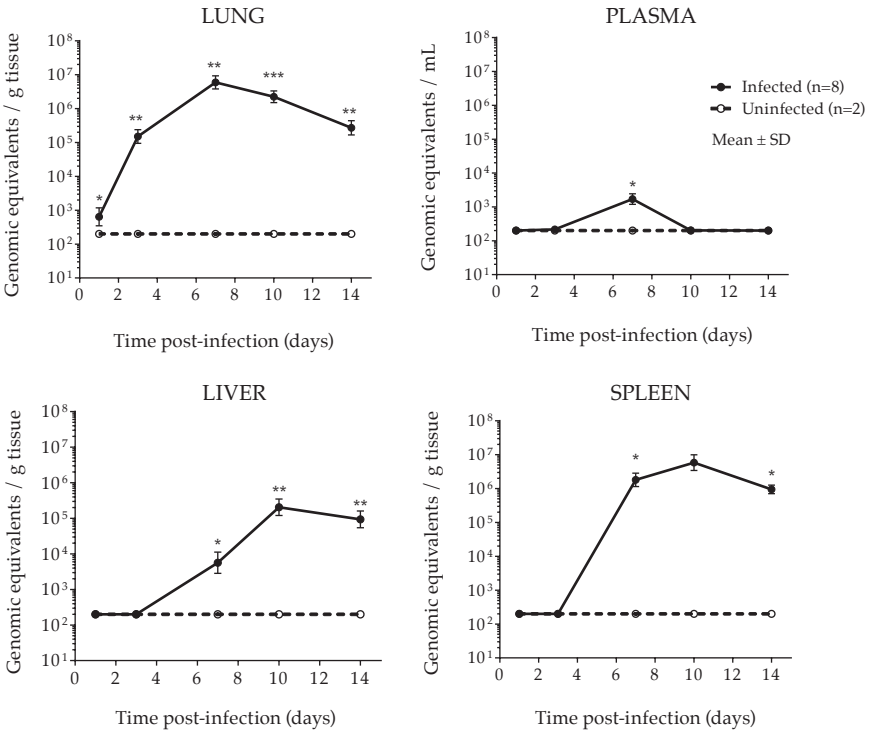

Figure 2. Number of *C. burnetii* DNA copies in lung, plasma, liver, and spleen after aerosol infection with *C. burnetii* in immunocompetent BALB/c mice. The mean ± SD numbers of genomic equivalents per gram of tissue or milliliter of plasma are shown of 8 infected mice per time point. Uninfected mice were negative at every time point in all tissues. Samples that were negative were assigned a value of 200 genomic equivalents per gram (or milliliter). This is the limit of detection of the assay. *P* values were calculated by 1-sample *t*-test with a hypothetical value of 0. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

similarly high in uninfected and infected mice. Unstimulated splenocytes produced some IFN-γ at day 10. NM-induced IFN-γ production, absent in all uninfected mice and in infected mice at days 1, 3, and 7, was significantly increased at day 10 and 14 postinfection.

Regarding the other cytokines, NM stimulation induced significant IL-6, KC, and IP-10 from day 10 onwards, while MCP-1 production and low levels of IL-10 were observed on day 14 (Fig. 5). In addition, con A-stimulated splenocytes showed significantly increased IL-6 and IP-10 production at day 14. Unstimulated splenocytes produced IL-6 at day 10, but otherwise, no substantial amounts of any other cytokines at any time point. IL-1β and TNF-α production were below

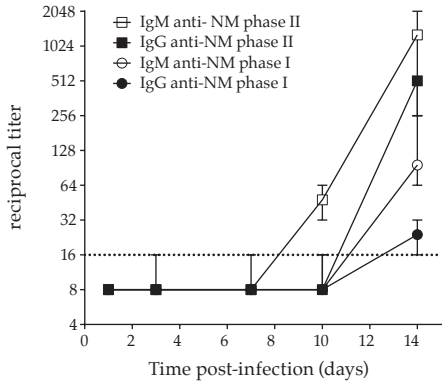

Figure 3. Antibody responses to *C. burnetii* after aerosol infection with *C. burnetii* in immuno-competent BALB/c mice. IgM and IgG titers to NM phase I and phase II were measured in plasma by IFA. The median ± range reciprocal titers are shown of 4 pairs of infected mice per time point. The control mice were seronegative at every time point (not shown). Negative results in the IFA were assigned a value 1:8.

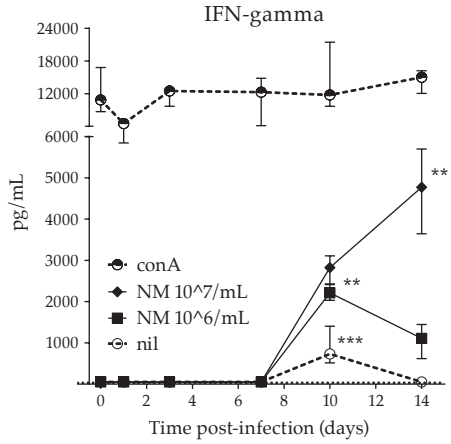

Figure 4. Early IFN-γ production by stimulated splenocytes of *C. burnetii*–infected immuno-competent BALB/c mice. Splenocytes were stimulated for 48 h with conA (2.5 μg/mL), NM phase I (10^7/mL), NM phase I (10^6/mL), or left unstimulated (nil). The median ± interquartile range cytokine production is shown per time point of 4 pairs of infected mice. T = 0 shows the median ± interquartile range of 5 pairs of uninfected mice. *P* values were calculated by Kruskal–Wallis test followed by Dunn's multiple comparison test comparing cytokine concentrations of infected mice at different time points with uninfected mice.
$^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Abbreviations: conA = concanavalin A; NMI = *C. burnetii* Nine Mile phase I.

detection limits at all time points with all stimuli. Supplementary Table 1 contains all data on all the cytokines measured.

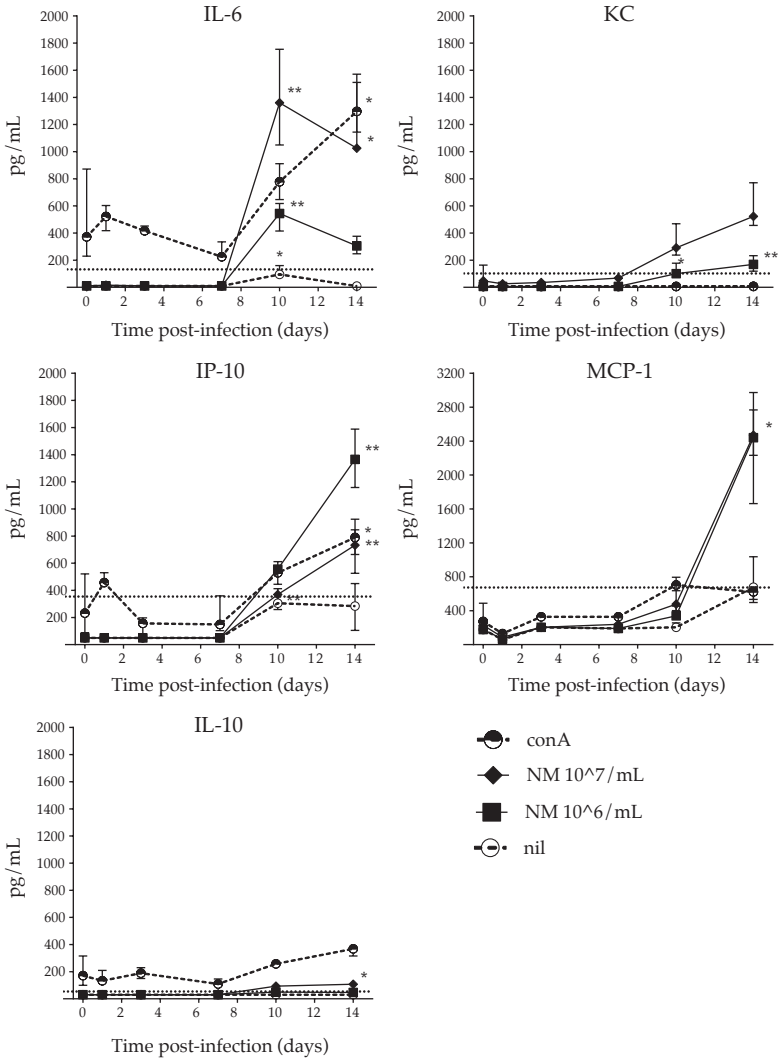

Figure 5. Early IL-6, KC,MCP-1, IP-10, and IL-10 production by stimulated splenocytes of *C. burnetii*–infected immunocompetent BALB/c mice. Splenocytes were stimulated for 48 h with conA (2.5 μg/mL), NM phase I (10^7/mL or 10^6/mL), or left unstimulated (nil). The median ± interquartile range cytokine production is shown per time point of 4 pairs of infected mice. T=0 shows the median ± interquartile range of 5 pairs of uninfected mice. IL-1β and TNF-α production were below the lowest detection limit at all time points for all stimulations (not shown). The dashed horizontal line represents the lowest standard in the Luminex assay; values below are extrapolated. *P* values were calculated by Kruskal–Wallis test followed by Dunn's multiple comparison test comparing cytokine concentrations of infected mice at different time points with uninfected mice. *P < 0.05, **P < 0.01. Abbreviations: conA = concanavalin A; NMI = *C. burnetii* Nine Mile phase I.

Discussion

In the present study, we observed effective early immune responses in immunocompetent BALB/c mice infected with *C. burnetii* via the aerosol route. After initial expansion of bacteria in lungs and spread to other tissues, the infection was cleared from day 10 onwards as reflected by the decreasing number of bacterial DNA copies. Antigen-induced IFN-γ production by splenocytes, indicating a cell-mediated immune response, coincided with emergence of IgM phase II antibodies at day 10 postinfection. This was accompanied by the production of proinflammatory cytokines including IL-6, KC, and IP-10, followed by MCP-1, but not by IL-1β and TNF-α, and only very low production of the anti-inflammatory cytokine IL-10.

Previous studies in humans have looked at the in vitro IFN-γ response in people vaccinated against Q fever, people that have had a previous Q fever infection, and chronic Q fever patients (Izzo and Marmion, 1993; Kersh et al., 2013; Limonard et al., 2012; Schoffelen et al., 2013a, 2013b, 2014). However, the testing of in vitro IFN-γ responses in acute human Q fever has not been reported. Acute cytokine responses in mice have been studied previously. This has been done by infecting mice by either an intraperitoneal or intratracheal route and then measuring circulating cytokines in serum at single time points (Russell-Lodrigue et al., 2009). These studies have detected IFN-γ and other cytokines in mouse serum but have not looked at in vitro antigen-specific recall responses at different time points. Another study looked at some early time points and detected modest increases in IFN-γ recall responses after infection of mice by intraperitoneal or intravenous routes (Honstettre et al., 2006). The study reported here describes in vitro antigen-specific recall cytokine responses at multiple time points shortly after *C. burnetii* aerosol infection. The data show a very robust antigen-specific IFN-γ response that is detected at about the same time as antibody responses relative to infection and perhaps just prior to detection of *C. burnetii*–specific IgG. Based these observations, specific IFN-γ production assays are worthwhile to investigate for detection of human acute Q fever.

The aerosol infection route that was used in this study closely reflects the typical acquisition of human infection by inhalation. After an inoculum of 6.8×10^4 bacteria, the number of *C. burnetii* DNA copies first increased in the lungs, after which the bacteria became detectable in plasma, with subsequent spread to the spleen and liver.

After the initial expansion, bacterial numbers declined. Since we aimed to observe the very early immune response, later time points were not studied. A previous study of intratracheal infection with *C. burnetii* in BALB/c mice that continued to 24 days postinfection also showed clearance of infection (Read et al., 2010). In that study, the genome copy numbers in the lungs sharply decreased between days 9

and 16, similar to our observations. Moreover, the spleen weight peaked at day 16 postinfection but had decreased by day 24, another indication that infection was controlled.

The time–response curves of the humoral immune response suggest that IgG antibodies are redundant for early clearance of *C. burnetii*. Due to the absence of cellular IgM receptors, IgM by itself—in contrast to IgG—is unable to influence cellular responses. In our model, IgG, against either phase I or phase II bacteria, became detectable only at day 14, whereas the clearance of bacteria from the lungs started between days 7 and 10. The decrease in bacterial DNA occurred, however, simultaneously with the increase of specific IFN-γ production by splenocytes.

A limitation of present study is that the results of cytokine production upon stimulation with recall antigens were obtained in splenocytes instead of peripheral blood cells, which would likely be used for testing in humans. Although there is not a specific reason to believe that IFN-γ production by peripheral blood cells would be completely different from splenocytes, there could be kinetic differences. In present study, stimulation of mouse whole blood in vitro was performed, but cytokine responses were difficult to detect due to technical limitations of whole blood stimulations in mice. IFN-γ could not be detected, while IP-10 was detectable only in low levels showing maximum levels at day 10 (not shown). Previous studies looking at past infection or vaccination against Q fever have found that human peripheral blood is a good source of cytokine-producing cells in response to *C. burnetii* antigen stimulation (Schoffelen et al., 2013a, 2013b).

In addition to IFN-γ, we observed the production of IL-6, KC, MCP-1, and IP-10 in *C. burnetii* stimulated splenocytes at days 10 and 14 after infection. These cytokines probably play an important role in the cell infiltration in *C. burnetii*–infected tissues of immunocompetent BALB/c mice, which was observed by Read et al. (2010). The increased production of IL-6 has been described in the course of human acute Q fever, in which unstimulated peripheral blood cells showed increased production of pro-inflammatory cytokines including IL-6, TNF-α, IL-12, and the anti-inflammatory cytokine IL-10 (Honstettre et al., 2003). However, IL-6 production by *C. burnetii*–stimulated blood cells was not increased in acute Q fever patients. Likewise, MCP-1 was found to be increased in unstimulated blood cells of acute Q fever patients, but not in *C. burnetii*–stimulated cells (Meghari et al., 2006). These human patients were, however, in a later stage of the infection than the mice in the current study.

To our knowledge, IP-10 has not been studied in the context of *C. burnetii* infections before. IP-10 is a chemokine produced by monocytes/macrophages, mainly in response to IFN-γ but also other cytokines including type I interferons, IL-2, IL-23, and IL-17. IP-10 has shown a promising role as an additional marker of *Mycobacterium tuberculosis* infection (Ruhwald et al., 2012), being specifically

induced by tuberculosis (TB)–specific antigens in confirmed TB cases and not in healthy controls. These data from human patients infected with an obligate intracellular pathogen are in line with our observations in mice infected with *C. burnetii*. However, Ruhwald et al. (2012) showed that IP-10 levels after antigen stimulation are higher compared with IFN-γ levels in human peripheral blood. In our study, we found that IP-10 was induced in antigen-stimulated splenocytes, but levels of IP-10 (1400 pg/mL) were lower than levels of IFN-γ (5000 pg/mL).

Previous studies have shown absent IL-1β production but substantial TNF-α production by *C. burnetii*–stimulated peritoneal macrophages of uninfected BALB/c mice (Ochoa-Repáraz et al., 2007). Other studies showed increased TNF-α production by peritoneal macrophages of *C. burnetii*–infected BALB/c mice (Honstettre et al., 2006). In our model, using the respiratory infection route, we were unable to induce substantial IL-1β and TNF-α production in stimulated spleen cells of infected mice. The lack of TNF-α response in our system could be due to the route of infection used, the cell types present in our spleen preparations, or the antigen used.

Of note, only minimal levels of IL-10 were produced by splenocytes after *C. burnetii* infection. IL-10 has been of special interest in previous studies, since it was linked to persistent infection in humans (Capo et al., 1996; Honstettre et al., 2003) and chronic Q fever in mice overexpressing IL-10 (Meghari et al., 2008). Our findings show that clearance of *C. burnetii* in the early stage is accompanied by only a very low antigen-specific IL-10 production by splenocytes. Earlier studies in intraperitoneally *C. burnetii*–infected BALB/c mice showed high levels of IL-10 production by *C. burnetii*–stimulated peritoneal macrophages at day 7 postinfection, before bacterial load decreased (Honstettre et al., 2006). Similar to TNF-α, macrophages may be an important source of IL-10 in response to *C. burnetii*, although it is likely that regulatory T cells and Th2 lymphocytes also play a role.

In conclusion, the model of *C. burnetii* infection used in this study demonstrates that detection of antigen-induced IFN-γ could be used to detect acute *C. burnetii* infection in mice, and this is likely to be the case in humans as well. Antigen-specific production of IFN-γ and IP-10 were both detectable prior to elevation of specific IgG antibodies. This study also showed antigen-specific induction of IL-6, KC, and MCP-1 from splenocyte cultures. If applied to immune-deficient mice or mice with anatomical risk factors for endocarditis or vascular infections, the model may offer wide opportunities to study the pathophysiological and immune derangements that occur during progression from acute to chronic Q fever.

Supplementary data to this article can be found online at http://dx. doi.org/10.1016/j.diagmicrobio.2014.12.008.

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III

Immunosuppressive drugs for rheumatoid arthritis as a risk factor for chronic Q fever

10

The risk of chronic Q fever in rheumatoid arthritis patients with and without anti-TNFα therapy

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Abstract

Objectives: During the recent Dutch Q fever epidemic, many individuals became infected with the intracellular bacterium *Coxiella burnetii*. Initial infection is often asymptomatic, but can be complicated by chronic Q fever in 1-5% of cases, months to years later. We examined whether rheumatoid arthritis **(**RA) patients on anti-tumor necrosis factor α (anti-TNFα) therapy are at increased risk for chronic Q fever.

Methods: RA patients, living in the Q fever endemic area, were screened for anti-*C. burnetii* antibodies in two cohorts: 1. RA patients on anti-TNFα therapy and 2. anti-TNFα naive RA patients. Patients with phase I and/or II IgG titres ≥1:32 were defined as seropositives, indicating previous exposure to *C. burnetii*. Chronic Q fever was diagnosed by a team of medical specialists.

Results: 57/361 (15.8%) patients on anti-TNFα therapy were seropositive, compared to 55/398 (13.8%) anti-TNFα naive patients (P=0.47). 10/112 (8.9%) seropositive patients were diagnosed with chronic Q fever: 7/57 (12.3%) patients on anti-TNFα therapy and 3/55 (5.5%) anti-TNFα naive patients (RR 2.25; 95% CI 0.61–8.27, P=0.32). Univariate analysis in all seropositive patients identified higher age, cardiac valvulopathy/prosthetic valve or aneurysm/vascular prosthesis and corticosteroid use as significant risk factors for chronic Q fever.

Conclusions: The prevalence of chronic Q fever in seropositive RA patients was higher (8.9%) than previously reported in infected individuals in the general population, suggesting that patients with RA are at risk for development of chronic Q fever. However, we found no significant additional risk of anti-TNFα therapy.

Introduction

Q fever is a zoonosis caused by the intracellular bacterium *Coxiella burnetii*. During the Dutch Q fever outbreak (2007-2010) an estimate of more than 40,000 individuals became infected [1,2]. Initial infection leads to a mostly self-limiting febrile disease in approximately 40% of cases; however, in at least 60% it remains asymptomatic and often unrecognized [3]. Chronic infection, mainly manifesting as endocarditis or vascular infection, presents in 1-5% of individuals months to years after initial infection [4,5]. These conditions are often fatal if left untreated and should therefore be diagnosed in an early stage. Individuals most at risk are those with pre-existing cardiac valvular disease or vascular aneurysm or prosthesis [6-10]. Immunosuppression is another stated risk factor for chronic fever, although definition of the type of immunosuppression is poor and statistical empowerment of the increased risk for chronic Q fever in immunocompromised patients is absent. Case studies describe chronic Q fever in patients with (haematologic) malignancies, or with liver- and bone marrow transplantation [11,12]. In addition, a retrospective study of 102 Q fever endocarditis patients, reveals cancer as a risk factor, although the authors do not specify the use of immuno-modulating drugs[9]. An association between Q fever and the use of immunosuppressive drugs, including corticosteroids and methotrexate, is mentioned in three case reports: two with acute Q fever [13,14], one with Q fever endocarditis [15].

Tumor necrosis factor-α (TNFα) plays an important role in the defense against intracellular bacteria such as *C. burnetii* [16]: In-vitro studies show that TNFα mediates interferon-gamma-induced intracellular killing of *C. burnetii* in monocytes through apoptosis [17], while neutralizing TNFα in-vitro decreases *C. burnetii* internalization in monocytes of Q fever endocarditis patients [18], and TNFα knock-out mice infected with *C. burnetii* develop early bacteraemia and severe heart lesions [19]. On the other hand, overproduction of TNFα by blood mononuclear cells of acute Q fever patients with valvulopathy is associated with development of chronic Q fever [20], and monocytes of Q fever endocarditis patients produce more TNFα than cells from cured patients or immunologically naive individuals.

Anti-TNFα therapy increases the risk of infections with intracellular bacteria such as mycobacterium tuberculosis [21,22]. Clinical data on the risk of *C. burnetii* infection in patients on anti-TNFα therapy are lacking. In view of the widespread use of TNFα-blockers in patients with rheumatoid arthritis (RA) or other inflammatory conditions, and the severe consequences of chronic Q fever, we decided to perform a controlled study to examine whether RA patients on anti-TNFα therapy are at increased risk of chronic Q fever. The regional Q fever

outbreak situation in the Netherlands served as a unique opportunity to perform such a study.

Methods

Patient enrollment

RA patients were selected from the rheumatology outpatient clinic of the Jeroen Bosch Hospital in 's- Hertogenbosch, the Radboud University Nijmegen Medical Centre and the Sint Maartenskliniek in Nijmegen. All three hospitals are located in or near the Q fever epidemic area. The study was approved by the local medical ethics review committee in Nijmegen. All patients lived in the Q fever epidemic area at the time of inclusion according to postal code [23].

Two cohorts were selected; The first cohort (anti-TNFα treated group) consisted of patients using TNFα-blockers, including adalimumab, etanercept and infliximab, for at least three months during the Q fever outbreak (between January 1st 2007 and January 1st 2010). The second cohort (anti-TNFα naive controls) consisted of patients who did not use any of these TNFα-blockers or other biological disease modifying anti-rheumatic drugs (biological DMARDs) in this time frame nor thereafter.

Exclusion criteria for both cohorts were age under 18 years, current pregnancy, lactation, lymphoproliferative disease and haematologic malignancies, chronic infections with HIV, hepatitis B or C, active mycobacterial disease and legal incapability.

Medication details and medical history were retrieved from patients' records and collected in an additional questionnaire.

Q fever diagnostics and disease definitions

From all participating patients, blood samples were obtained between December 2011 and June 2012. Serum was screened for IgG against both *C. burnetii* antigenic phases, phase I and II, by means of immunofluorescence assay (IFA; Focus Diagnostics, Cypress, CA, USA), using a cutoff titre of ≥1:32. If screening was above this cutoff, exact titres of IgG and IgM antibodies against *C. burnetii* phase I and II were determined. In case of a phase I IgG ≥1:512, PCR for *C. burnetii* DNA was performed on plasma. Subjects were classified as seropositives when *C. burnetii* IgG antibodies were ≥1:32. All seropositive individuals were followed-up three to six monthly in the year after the initial screening by their rheumatologist or an infectious disease specialist. The end of the study-period was defined as one year after the last inclusion. Seropositive individuals with suspected chronic Q fever underwent further medical assessment including imaging, i.e. echocardiography and whole body positron emission tomography-computed tomography (PET-CT).

Chronic Q fever was diagnosed or excluded by a team of medical specialists, who were guided by the Dutch consensus on chronic Q fever diagnostics [24]. Chronic Q fever was considered as proven if PCR was positive, or if phase I IgG was ≥1:1024 and endocardial involvement, large vessel or prosthetic infection was confirmed by imaging. All other chronic Q fever cases were classified as probable, since all had at least one stated risk factor, e.g. immunocompromised status. Past acute *C. burnetii* infection was defined by seropositivity at enrollment without the diagnosis of chronic Q fever during one year follow-up.

To prevent information bias, the cause of death was retrieved from the medical records of patients that had deceased after January 1st 2007 but before study enrollment, and possible relation of death to Q fever was determined. Patients with a known chronic Q fever infection who died of acute aneurysms or acute heart failure, were identified to have a cause of death 'related to Q fever'. Patients who died of acute aneurysm, complications of vascular prosthesis, endocarditis or acute heart failure, but did not have a known chronic Q fever infection, were identified as patients with a cause of death 'possibly related to Q fever'.

Statistical analyses

Clinical and microbiological data for all subjects were collected and analyzed in an SPSS database (version 18.0). For comparison of the baseline characteristics, data were expressed as mean values or percentages. Mean values were compared using independent samples *t*-test. Percentages were compared using Fisher's exact test. The proportion seropositive patients and chronic Q fever patients was compared between both groups and the relative risk (RR) with 95% confidence intervals (95% CI) were calculated. In an univariate analysis of all seropositive patients, we aimed to identify other possible risk factors for chronic Q fever. Multivariate analysis could not be performed, due to the low number of cases. In all analyses, $P<0.05$ was considered significant.

Results

Study population

A total of 1319 RA patients were found eligible for inclusion in this study. Of the 1319 selected patients, 86 had died before study enrollment but after January 1st 2007. The cause of death of 56 of these patients could be retrieved from the medical records; none was rated related to Q fever, only four were possibly related.

The 1233 remaining living patients were invited for microbiological screening. The response rate was 759/1233 (62%). Of the included subjects, 361 used TNFα-blockers for at least three months during the Q fever outbreak, while 398 were

anti-TNFα naive. Table 1 shows the baseline characteristics of the two cohorts. The cohort on anti-TNFα therapy during the epidemic was younger, and used significantly more often corticosteroids during the epidemic and currently (P<0.0001).

Diagnosis of Q fever in the anti-TNFα cohort and the anti-TNFα naive cohort

In total, 112/759 (14.8%) patients were seropositive: 57/361 (15.8%) in the anti-TNFα cohort and 55/398 (13.8%) in the anti-TNFα naive cohort, which was not significantly different (*P*=0.47). Three selected patients (two in the anti-TNFα therapy cohort and one in the anti-TNFα naive cohort) were already diagnosed with chronic Q fever before enrollment in this study.

Overall, 10/112 (8.9%) seropositive patients were diagnosed with chronic Q fever: seven in the anti- TNFα group and three in the anti-TNFα naive group (see Table 2 for details). Thus, 12.3% (7/57) of the seropositive RA patients using anti-TNFα had progressed into chronic Q fever after contact with *C. burnetii*, and 5.5% (3/55) of the anti-TNF naive patients (RR 2.25; 95% CI 0.61–8.27, *P*=0.32). Three of the ten patients had known pre-existing valvular and/or vascular risk factors for chronic Q fever, while the others had none. All were offered transesophageal echocardiogram (TEE); three patients refused TEE, yet underwent transthoracic echocardiogram (TTE). Two had echocardiographic signs of endocarditis. Two showed abnormalities on PET-CT, which revealed increased uptake in aortic valve in one and increased uptake in pleural effusion in another. Two patients treated for chronic Q fever had IgG titres against phase I below the threshold of 1:1024, one of whom had a positive *Coxiella* PCR in pleural effusion. The other was diagnosed with probable chronic Q fever based on fatigue and newly diagnosed abnormalities of the aortic valve on TEE. Blood cultures were negative. Although chronic *C. burnetii* infection could not be confirmed by PCR on blood (and no cardiac valve tissue was available, since cardiac surgery was not performed sofar), longterm antibiotic treatment for chronic Q fever was started in this patient.

Serological follow-up of seropositives

In the remaining 102 seropositive patients, no chronic Q fever was diagnosed in the follow-up period of one year. Upon screening, all of them had IgG against phase II antibodies. The serologic screening results are shown separately for the anti-TNFα cohort and the anti-TNFα naive cohort in table 3. The geometric mean titres of IgG against phase I and phase II were not significantly different between both groups (*P*=0.28 and *P*=0.061 respectively).

Table 1 Baseline characteristics of all included patients.

	Anti-TNF group n = 361 (%)	**Anti-TNF naive group n = 398 (%)**	**p-value**
Mean age ± SD[a]	59.9 (±11.0)	62.1 (±12.2)	**0.01**
Male sex	109 (30.2)	128 (32.2)	0.58
Valvulopathy / prosthetic valve	14 (2.2)	19 (4.8)	0.60
Vascular aneurysm or prosthesis	4 (1.1)	8 (2.0)	0.39
Ischaemic heart disease	43 (11.9)	53 (13.3)	0.59
Peripheral vascular disease	7 (1.9)	15 (3.8)	0.19
Cerebral vascular disease	17 (4.7)	25 (6.3)	0.43
Dyslipidaemia	65 (18.0)	86 (21.6)	0.24
Hypertension	110 (30.5)	139 (35.0)	0.22
Diabetes mellitus[b]	27 (7.5)	45 (11.3)	0.08
Pregnancy (between 2007-2010)[c]	5 (1.4)	3 (0.8)	0.49
Malignancy[d]	8 (2.2)	14 (3.5)	0.39
Renal insufficiency[e]	19 (5.3)	26 (6.5)	0.54
Use of TNF-α blocker during epidemic[f]	361 (100)	-	n/a
Use of etanercept during epidemic	194 (53.6)	-	n/a
Use of adalimumab during epidemic	148 (40.9)	-	n/a
Use of infliximab during epidemic	70 (19.3)	-	n/a
Months of TNF-α blocker use (mean ±SD)[g]	26.0 (±11.6)	-	n/a
Use of DMARD[h] during epidemic	283 (78.2)	397 (100.0)	n/a
Use of methotrexate during epidemic	225 (62.3)	297 (74.6)	n/a
Months of DMARD use (mean ±SD)	24.4 (±15.2)	30.7 (±9.5)	n/a
Use of corticosteroids during epidemic	103 (28.5)	63 (15.8)	**0.000**
Months of corticosteroid use (mean ±SD)	7.3 (±13.2)	3.2 (±9.1)	**0.000**
Use of DMARD or / and corticosteroids during epidemic	307 (85.0)	397 (100.0)	n/a
Use of other biological during epidemic	29 (8.0)	-	n/a
Current use of TNF-α blocker	256 (70.9)	-	n/a
Current use of DMARD	248 (68.7)	366 (92.0)	n/a
Current use of corticosteroids	81 (22.4)	40 (10.1)	**0.000**
Current use of other biological[i]	61 (16.9)	-	n/a

SD, standard deviation; n/a, not applicable; DMARD, disease modifying anti-rheumatic drug

[a] Age at November 1st 2011.

[b] Diabetes mellitus (DM) is defined as DM type 1 or DM type 2.

[c] Pregnant patients did not receive anti-TNF treatment during pregnancy.

[d] Patients with a malignancy diagnosed after January 1st 2007, patients that were treated after January 1st 2007 for malignancy or with metastatic disease. Basocellular carcinoma are not included because of diminutive clinical impact. There were no patients with haematological malignancies included in this study.

[e] Renal insufficiency is defined as a GFR <60/min.

[f] Epidemic is defined as the outbreak between January 1st 2007 and January 1st 2010 in the Netherlands.

[g] During the epidemic as defined; maximum of 36 months.

[h] Use of DMARD: not including use of corticosteroids.

[i] Other biologicals were golimumab, certolizumab, abatacept, anakinra, rituximab, tocilizumab.

Table 2 Results of microbiological analysis and clinical assessment at initial screening and 2-4 months of follow-up of 10 patients diagnosed with probable or proven chronic Q fever.

			Initial results			
	Sex	**Cohort**	**IgG titre against phase I**	**IgG titre against phase II**	**PCR in blood**	**Valvular or vascular risk factors for chronic Q fever[a]**
1	Male	Anti-TNF	1:4096	1:4096	-	Aortic valve stenosis, abdominal aortic aneurysm
2	Female	Anti-TNF	1:8192	1:8192	+	Mitral valve repair
3	Male	Anti-TNF	1:16384	1:16384	-	None
4	Female	Anti-TNF	1:1024	1:1024	-	None
5	Male	Anti-TNF	1:1024	1:4096	-	None
6	Female	Anti-TNF	1:1024	1:2048	-	None
7	Female	Anti-TNF	1:256	1:512	-	None
8	Male	TNF-naive	1:512	1:512	-	None
9	Male	TNF-naive	1:2048	1:1024	-	Vascular prosthesis abdominal aorta
10	male	TNF-naive	1:2048	1:2048	-	None

PCR, polymerase-chain reaction; TNF, tumor necrosis factor alpha. [a] valvulopathy, valvular prosthesis, aneurysms, vascular prosthesis. [b] during epidemic, at least 3 months. [c] based on anamnesis, physical examination and imaging (echocardiogram and whole body positron emission tomography-computed tomography). [d] in addition to surgical repair of endoleak vascular prosthesis.

Univariate analysis of risk factors for chronic Q fever

To identify risk factors other than anti-TNFα treatment for development of chronic Q fever in RA, univariate analysis was performed, comparing the ten chronic Q fever cases with the 102 patients with past *C. burnetii* infection. Clinical data, anti-rheumatic medication use during Q fever epidemic, and known risk factors for chronic Q fever are listed for both groups in table 4. The RA patients with chronic Q fever were significantly older than those who did not develop chronic Q fever (P=0.02) and, as expected, had significantly more often pre-existing vascular or valvular risk factors for chronic Q fever (P=0.05). Moreover, corticosteroid use

Anti-rheumatic medication[b]	Clinical assessment[c]	Antibiotic treatment	Results after 2-4 mo follow- up		
			IgG titre against phase I	IgG titre against phase II	PCR
Etanercept, methotrexate, hydroxychloroquine, prednisone	Endocarditis	yes	1:1024	1:512	-
Adalimumab, infliximab, prednisone	Endocarditis	yes	1:4096	1:4096	+
Infliximab, sulfasalazine, prednisone	Focus unclear	yes	1:8192	1:8192	-
Etanercept	Focus unclear	no	1:1024	1:1024	-
Etanercept, methotrexate, leflunomide, prednisone	Focus unclear	yes	1:2048	1:2048	-
Adalimumab, methotrexate	Focus unclear	no	1:256	1:4096	-
Adalimumab, sulfasalazine	Endocarditis	yes	1:512	1:1024	-
Methotrexate, sulfasalazine	PCR+ pleural effusion	yes	1:512	1:512	-
Methotrexate, prednisone	Vascular prosthesis infection	yes[d]	1:2048	1:1024	-
Methotrexate, prednisone	Focus unclear	no	1:1024	1:1024	-

during the epidemic was identified as a predisposing factor for development of chronic Q fever (P=0.02), as well as its duration of use (P=0.002). The use of metho--trexate, any DMARD or any biological DMARD (other than infliximab, adalimumab or etanercept) were not found to be a significant predisposing riskfactor.

Table 3 Titres of IgG against phase I and II *C. burnetii* in anti-*C. burnetii* antibody-positive RA patients without development of chronic Q fever; either with or without anti-TNF treatment during the Q fever epidemic.

	Anti-TNF treated RA patients (n=50)		TNF-naive RA patients (n=52)	
	IgG against phase I	**IgG against phase II**	**IgG against phase I**	**IgG against phase II**
Negative	22 (44.0)	0 (0.0)	26 (50.0)	0 (0.0)
1:32	6 (12.0)	3 (6.0)	9 (17.3)	13 (25.0)
1:64	10 (20.0)	10 (20.0)	7 (13.5)	7 (13.5)
1:128	5 (10.0)	13 (26.0)	7 (13.5)	10 (19.2)
1:256	7 (14.0)	10 (20.0)	3 (5.8)	14 (26.9)
1:512	0 (0.0)	8 (16.0)	0 (0.0)	4 (7.7)
1:1024	0 (0.0)	4 (8.0)	0 (0.0)	4 (7.7)
1:2048	0 (0.0)	2 (4.0)	0 (0.0)	0 (0.0)
≥1:4096	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

RA, rheumatoid arthritis; TNF, tumor necrosis factor alpha. No. (%) of patients is indicated.

Discussion

To our knowledge, this is the first systematic study on the risk on chronic Q fever in a clearly defined immunocompromised patient group living in an epidemic area. We found a prevalence of Q fever seropositivity in RA patients of 14.8%, approximately the same as the 12.2% previously reported for the general population living in the same area.[25] Despite this, the overall prevalence of chronic Q fever among seropositive RA patients was 8.9%, which is higher than what is described by others in an unselected population (1-5%). These figures suggest that RA patients may not be more susceptible for initial Q fever infection, but may be more prone for the development of chronic Q fever. However, in RA patients on anti-TNFα therapy, no significant additional risk for chronic Q fever was found (RR 2.25; 95% CI 0.61–8.27, P=0.32). In this respect, we were unable to show that anti-TNFα therapy increases the risk for progression to chronic Q fever.

An important finding of this study was a prevalence of chronic Q fever of 8.9% among *C. burnetii* seropositive RA patients, which is relatively high. Since we did

Table 4 Univariate analysis of risk factors for development of chronic Q fever in all Q fever seropositive RA patients.

	Chronic Q fever infection n =10 (%)	Past Q fever infection n = 102 (%)	p-value
Mean age ± SD (yr)	66.3 ± 8.5	58.3 ± 10.6	**0.02**
Male Sex	6 (60)	47 (46.1)	0.51
Vascular aneurysm / prosthesis or valvulopathy / prosthetic valve[a]	3 (30)	7 (6.9)	**0.05**
Current use of TNF-α blocker	4 (40)	40 (39.2)	1.00
TNF-α blocker during epidemic[b]	7 (70)	50 (49.0)	0.32
TNF-α blocker use > 1 year during epidemic	5 (50)	41 (40.2)	0.74
Months of TNF-α blocker use (mean ±SD)[c]	16.6 (±15.7)	12.5 (±15.1)	0.41
Current use of corticosteroids	3 (30)	13 (12.7)	0.15
Corticosteroids during epidemic	6 (60)	23 (22.5)	**0.02**
Corticosteroid use > 1 year during epidemic	4 (40)	15 (14.7)	0.06
Months of corticosteroid use (mean ±SD)	15.8 (±17.6)	4.3 (±9.9)	**0.002**
Current use of DMARD[d]	8 (80)	87 (85.3)	0.65
DMARD during epidemic	8 (80)	95 (93.1)	0.18
Methotrexate during epidemic	6 (60)	75 (73.5)	0.46
DMARD use > 1 year during epidemic	8 (80)	84 (82.4)	1.00
Methotrexate use > 1 year	6 (60)	67 (65.7)	0.74
Months of DMARD use (mean ±SD)	27.5 (±15.1)	27.5 (±12.3)	0.99
Months of methotrexate use (mean ±SD)	20.3 (±17.9)	21.6 (±15.7)	0.81
Current use of other biological[e]	1 (10)	5 (4.9)	0.44
Other biological during epidemic	0 (0)	5 (4.9)	1.00

RA, rheumatoid arthritis; DMARD, disease modifying anti-rheumatic drug; SD, standard deviation
[a] Known risk factors for progression to chronic Q fever infection.
[b] Epidemic is defined as the outbreak between 1-1-2007 and 1-1- 2010 in the Netherlands.
[c] During epidemic as defined; maximum of 36 months.
[d] Use of DMARD does not include use of corticosteroids.
[e] Other biologicals were golimumab, certolizumab, abatacept, anakinra, rituximab, tocilizumab.

not include a control cohort without RA and immunosuppressive medication in our study, the interpretation must be done cautiously. A previous study conducted in 2010 in 686 unselected individuals with an acute Q fever episode in 2007-2008,

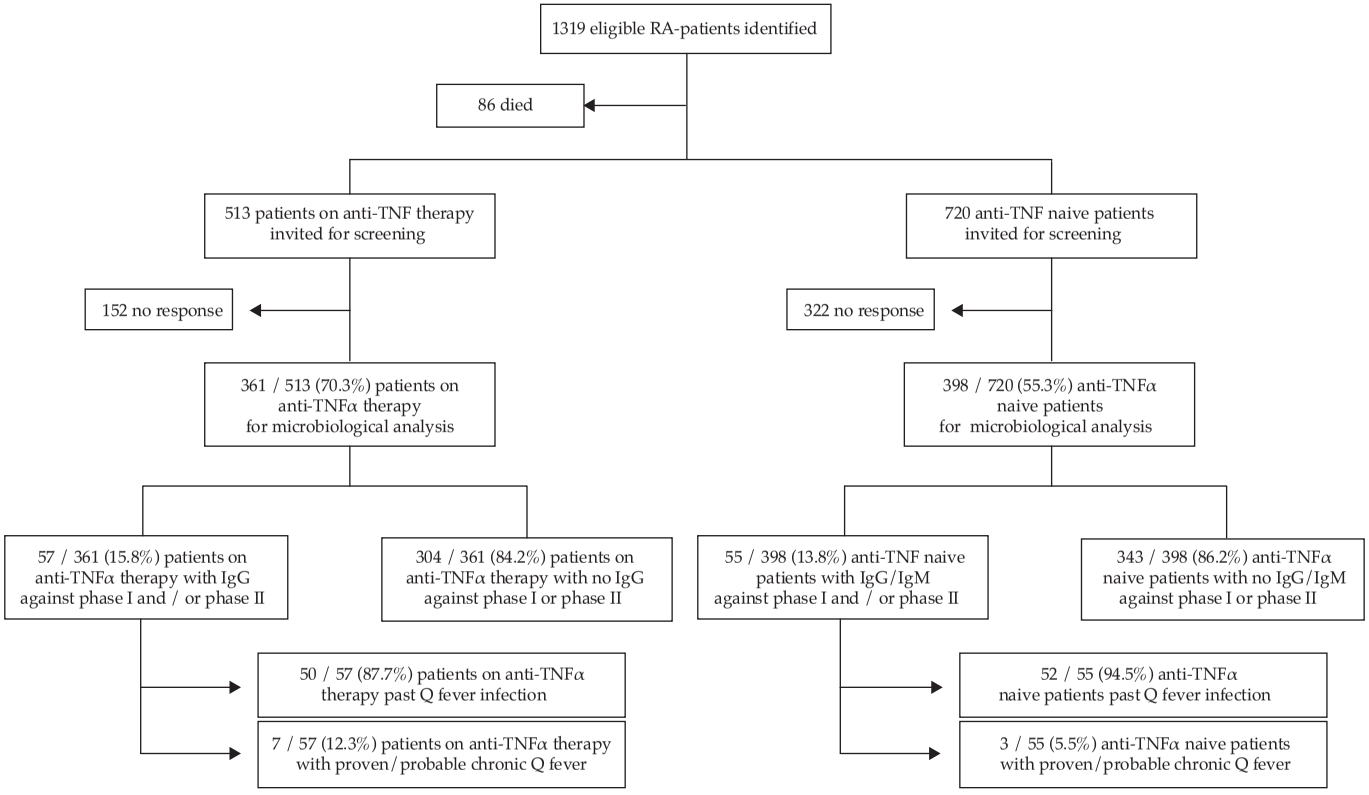

Figure 1. Flow diagram showing patient inclusion and Q fever screening results

living in the same area as our RA patients, showed a prevalence of chronic Q fever of 1.6% [26]. Considering this population to be a historic control cohort, the prevalence of chronic Q fever in our RA population is significantly higher ((RR 5.57; 95% CI 2.42–12.81, *P*=0.0002).

Although we found an increased risk for the development of chronic Q fever in the anti-TNFα treated group compared to the anti-TNFα naive group (12.3 vs 5.5%), this difference was not significant, mainly due to the low number of identified cases, which could have resulted in a type II error.

However, in univariate analysis, we found higher age and valvular/vascular defects or prosthesis to be risk factors for chronic Q fever, as described previously in a Dutch cohort of chronic Q fever patients [8], and the use of corticosteroids during the epidemic. Interestingly, the anti-TNFα group used significantly more often corticosteroids, which could partly explain the increased risk we found among anti-TNFα users. The finding of corticosteroids as a predisposing factor for chronic Q fever, could not be confirmed in multivariate analysis, because of the low number of chronic Q fever cases.

We expect to have detected most, if not all, of the chronic Q fever cases with our screening and follow-up strategy. The Q fever epidemics in the Netherlands subsided abruptly in 2010 after several veterinary interventions, and new infections after 2010 were extremely rare.[27] Our screening was performed 2-3 years after the peak of the Q fever epidemic and patients found to be seropositive at the start of the study were followed up for one year during which none progressed to chronic Q fever.

Even so, 75% of individuals who develop chronic Q fever after an acute Q fever episode, are diagnosed within one year [28]. Thus it is not to be expected that patients would develop chronic Q fever after the follow-up period.

We used the measurement of antibodies to detect Q fever. It should, however, be mentioned that in general immunosuppressive medication and immune-mediated diseases interfere with serologic responses, and antibody titres may be lower than in healthy individuals.[29] Influenza virus vaccination in anti-TNFα treated patients result in somewhat lower post-vaccination titres as compared to a control group on non-biological DMARDs [30,31]. Studies on pneumococcal polysaccharide vaccination, on the other hand, do not describe a negative impact of anti-TNFα use on vaccination outcomes [32]. Likewise, in our study, we did not find a significant difference in the level of anti-*C. burnetii* antibody titres between the seropositives in the anti-TNFα treated group and the anti-TNFα naive group. Therefore we assume that screening for antibodies is an appropriate tool to identify exposure to *C. burnetii* in our study groups, in spite of the background disease and medication.

Of note, the screening identified one case with proven and one case with probable chronic Q fever, with maximum phase I IgG titres of 1:512, just below the cutoff of 1:1024 [24]. The first patient used methotrexate and sulfasalazine during the Q fever epidemic and presented with a *C. burnetii* DNA positive pleural effusion. The second used adalimumab and sulfasalazine during the epidemic and was suspected for having Q fever endocarditis based on symptoms, elevated serum C-reactive protein and abnormalities on TEE. A recent publication showed that 4/57 patients with Q fever endocarditis, confirmed by *C. burnetii* PCR of cardiovascular surgical samples, had phase I IgG antibody titres ≤1:800 [33], but the authors do not mention the immune status of these four individuals. Moreover, in a Dutch cohort of chronic Q fever patients, 3/93 PCR-positive patients had a phase I IgG titre of 1:512 at diagnosis, one of whom used immunosuppressive agents [34]. All in all, this emphasizes that an absolute cutoff for phase I IgG antibody titres should not be handled to rigid, when other clues for infection are present. Elevated phase I IgG titres below the cutoff 1:1024 do not rule out the possibility of chronic Q fever in immunocompromised patients.

Interestingly, of the ten chronic Q fever RA patients identified in this study, only three patients had pre-existing vascular or valvular defects as risk factor for development of chronic Q fever. In fact, two of them were already diagnosed with chronic Q fever at inclusion while participating in a screening program of individuals with pre-existing cardiac valve abnormalities [6] and vascular defects. The finding that 70% of the patients with chronic Q fever had no pre-existing vascular or valvular defects, stresses the importance of immunodeficiency in the risk of developing chronic *C. burnetii* infection [12].

With regard to the pathophysiology of chronic *C. burnetii* infection, it remains unclear whether immunosuppression at the moment of exposure leads to direct progression to chronic infection or if starting immunosuppression leads to reactivation of a latent infection. The latter has been shown in guinea pigs, that experienced reactivation after recovery of previous *C. burnetii* infection caused by cortisone injections [35]. In tuberculosis both pathophysiological pathways are described, with active tuberculosis after start of anti-TNFα treatment interpreted as reactivation of latent infection, and tuberculosis longer after start of anti-TNF treatment interpreted as a new infection, sometimes immediately progressing to active disseminated disease [36]. In our study, the anti-TNFα cohort used TNFα-blockers with a mean duration of 26 months during the 3-year epidemic, and the anti-TNFα naive cohort used DMARDs with a mean duration of 31 months during the epidemic. This suggests that the majority of the initial *C. burnetii* infections coincided with the use of immunosuppressives.

In conclusion, our data suggests that RA patients are at increased risk for development of chronic Q fever. Therefore, careful evaluation for chronic Q fever is warranted after *C. burnetii* exposure in these patients. Since corticosteroid use during the outbreak was found in univariate analysis to be a specific risk factor for development of chronic Q fever, special attention should be paid to RA patients using corticosteroids. Anti-TNFα therapy, however, was not identified as an important risk factor for development of chronic Q fever.

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Acute and probable chronic Q fever during anti-TNFα and anti B-cell immunotherapy: a case report

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Abstract

Background: Q fever is caused by the intracellular bacterium *Coxiella burnetii*. Initial infection can present as acute Q fever, while a minority of infected individuals develops chronic Q fever endocarditis or vascular infection months to years after initial infection. Serology is an important diagnostic tool for both acute and chronic Q fever. However, since immunosuppressive drugs may hamper the humoral immune response, diagnosis of Q fever might be blurred when these drugs are used.

Case presentation: A 71-year-old Caucasian male was diagnosed with symptomatic acute Q fever (based on positive *C. burnetii* PCR followed by seroconversion) while using anti-tumor necrosis factor-α (anti-TNFα) drugs for rheumatoid arthritis (RA). He was treated for two weeks with moxifloxacin. After 24 months of follow-up, the diagnosis of probable chronic Q fever was established based on increasing anti-*C. burnetii* phase I IgG antibody titres in a immunocompromised patient combined with clinical suspicion of endocarditis. At the time of chronic Q fever diagnosis, he had been treated with anti B-cell therapy for 16 months. Antibiotic therapy consisting of 1.5 years doxycycline and hydroxychloroquine was started and successfully completed and no signs of relapse were seen after more than one year of follow-up.

Conclusion: The use of anti-TNFα agents for RA in the acute phase of Q fever did not hamper the *C. burnetii*-specific serological response as measured by immunofluorescence assay. However, in the presented case, an intact humoral response did not prevent progression to probable chronic *C. burnetii* infection, most likely because essential cellular immune responses were suppressed during the acute phase of the infection. Despite the start of anti-B-cell therapy with rituximab after the acute Q fever episode, an increase in anti-*C. burnetii* phase I IgG antibodies was observed, supporting the notion that *C. burnetii* specific CD20-negative memory B-cells are responsible for this rise in antibody titres.

Background

Q fever is caused by the intracellular growing bacterium *Coxiella burnetii* [1]. Acute Q fever is a (self-limiting) febrile illness, but can present as pneumonia or hepatitis. Chronic Q fever presents most often as an endovascular infection, i.e. endocarditis or mycotic aneurysm or infected vascular graft, which has a high mortality if left untreated [2,3]. Risk factors are underlying valvular defects, or pre-existing vascular aneurysm or prosthesis.

Immunosuppression is another stated risk factor for chronic Q fever, as some immunosuppressive drugs decrease protective cellular responses against intracellular growing bacteria. This risk factor has thus far been poorly documented, but recently we confirmed that patients with rheumatoid arthritis (RA) using immunosuppressive drugs are indeed at increased risk of developing chronic Q fever [4].

Clinical signs of *C. burnetii* infection are often nonspecific, and the diagnosis of acute or chronic Q fever is heavily based on measurement of antibody titres [5,6], complemented by the direct detection of the micro-organism by polymerase chain reaction (PCR) [7,8]. Serologic criteria for Q fever consist of measurement of antibodies against the two antigenic forms of *C. burnetii*, phase I and II organisms, with high anti-*C. burnetii* phase I IgG titres - in the absence of acute Q fever – pointing to a chronic infection. The appropriate cut-off titre that differentiates it from a past cleared infection is debated; currently proposed cut-offs are 1:1,024 or 1:1,600 [6,9].

The diagnosis of Q fever in hosts on immunosuppressive drugs may be complicated, because these drugs can inhibit antibody responses and therefore hamper correct diagnosis based on serologic results. Also the immunemediated disease itself, for which these drugs are prescribed, may contribute to inadequate immune responses to infection [10,11].

Here we present a case history of a patient with RA who had an episode of acute Q fever while being treated with anti-tumor necrosis factor-α (anti-TNFα) medication, and who developed probable chronic Q fever over the subsequent two years while using the anti-B-cell monoclonal antibody rituximab. The case highlights the importance of cellular and humoral immune response modifying agents in the natural course of *C. burnetii* infections and the possible pitfalls of the use of serological methods to detect the stage of disease.

Case presentation

Acute Q fever

In May 2009, during the Dutch Q fever epidemic, a 71-years-old rheumatoid factor and anti-CCP positive RA patient living in the Q fever high incidence area, presented

with 8 days of fever and a non-productive cough. He was receiving anti-rheumatic treatment including etanercept (an anti-tumor necrosis factor-α [anti-TNFα] agent) and prednisone. He had a history of atrial fibrillation, but no underlying valvulopathy. Physical examination and a chest X-ray were compatible with a pulmonary infiltrate. No murmurs were heard upon cardiac auscultation. Laboratory investigations revealed increased C-reactive protein (CRP, 285 mg/L), a normal leukocyte count (5.2×10^9/L) and normal values for renal function and liver enzymes. PCR (real-time PCR targeting the IS1111a insertion element [12]) for *C. burnetii* on plasma turned out to be positive. However, serology (immunofluorescence assay [IFA, Focus Diagnostics, Cypress, USA]) was negative for IgM as well as for IgG against phase I and II *Coxiella burnetii*. The diagnosis of acute Q fever was made and treatment with moxifloxacin 400 mg daily for 14 days was started. Two weeks later, seroconversion was observed with anti-phase I and II IgM titres of 1:4096 and 1:16384 respectively.

Diagnosis and treatment of probable chronic Q fever

After quick recovery, the patient was followed-up to monitor for possible progression to chronic Q fever. During this period, the anti-rheumatic treatment had been switched by the rheumatologist from etanercept to adalimumab (another anti-TNFα agent), and subsequently – 8 months after the acute Q fever episode – to rituximab. The latter, an anti-CD20 anti B-cell monoclonal antibody, had resulted in adequate suppression of the rheumatic activity.

As can be seen in Figure 1, anti-phase I IgG antibodies titres had not decreased below 1:1024 after more than one year, and continued to increase to 1:4096 at 24 months, suggesting development of chronic Q fever. At that moment, the patient had complaints of general fatigue but no fever, night-sweats or cardiac problems. On physical examination, however, a grade 2/6 aortic systolic murmur was audible. Transesophageal echocardiography (TEE) showed no signs of endocarditis, but an echogenic mitral annulus and a trace of mitral valve insufficiency. Laboratory investigation showed an ESR 40 mm/hr with CRP < 2 mg/L. PCR for *C. burnetii* in plasma was repeatedly negative. Abdominal ultrasound did not reveal an aortic aneurysm. Positron-emission tomography (PET)-scanning showed hilar lymph-adenopathy but no other abnormalties. Endobronchial biopsy of the hilar lymph nodes was PCR negative for *C. burnetii* and showed no signs of malignancy. Because of the increasing anti-phase I IgG titres in this immunocompromised patient, in combination with nonspecific complaints and the new cardiac murmur, the diagnosis of 'probable chronic Q fever' was made [13] and the patient was started on doxycycline 200 mg daily combined with hydroxychloroquine 200 mg three times daily for 1.5 years. Intermittent courses with rituximab were continued as anti-rheumatic treatment, in combination with azathioprine. During antibiotic

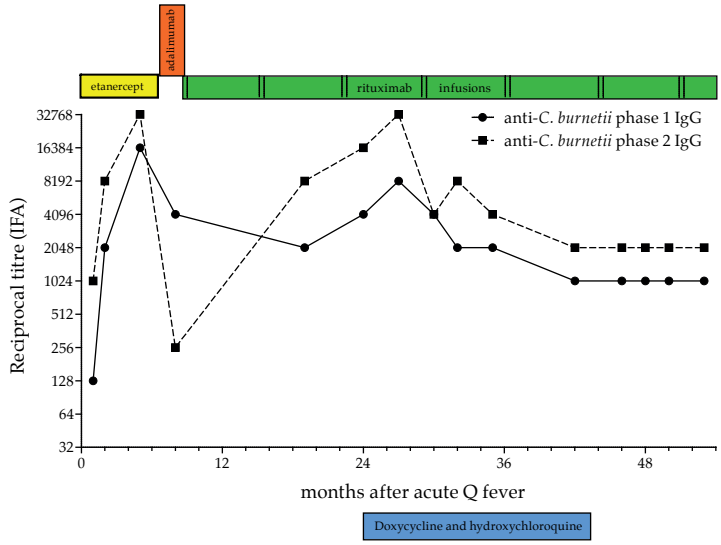

Figure 1. Q fever serology during follow-up after acute Q fever in a patient with anti-rheumatic drugs. Serological titres of anti-*Coxiella burnetii* phase I and phase II IgG (as measured by immunofluorescence assay) during follow-up after acute Q fever (t = 0) of a patient using subsequently etanercept, adalimumab and rituximab as biological disease-modifying anti-rheumatic drugs. Rituximab was given with 7 months intervals, each time two dosages with two weeks interval. At t = 24 the diagnosis probable chronic Q fever was established followed by anti-microbial treatment with doxycycline and hydroxychloroquine for 1.5 years.

treatment, the antiphase I IgG titres decreased from 1:8192 to 1:1024. The patient experienced improvement from his fatigue. After 1.5 years treatment, PET-scanning and TEE were unchanged. After discontinuation of antibiotics, the serological and clinical follow-up was pursued and is still continuing, with no relapse after more than one year.

Discussion

We report on an immunocompromised patient, followed-up from the start of a symptomatic acute Q fever episode to the development and treatment of probable chronic Q fever. Our case illustrates three interesting aspects of diagnosis and treatment of Q fever in an immunocompromised host. First, we noticed that, despite the presence of RA and use of anti-TNFα agents, the humoral response to the initial *C. burnetii* infection was not impaired. Secondly, in spite of adequate treatment, the acute Q fever progressed to probable chronic Q fever endocarditis,

which suggests incomplete clearance of the infection during the acute stage. Finally, the increase in phase I IgG titres, which serves as a marker for chronic Q fever, occurred under treatment with anti B-cell immunotherapy which was started after the acute Q fever episode.

In the presented case, a definite diagnosis of chronic Q fever could not be made. *C. burnetii* DNA was not detected in blood on several occasions and echocardiography did not show major signs of endocarditis. Nevertheless, chronic Q fever was clinically highly suspected. The patient was followed-up after acute Q fever, and we observed that the antibody titres were falling after they had peaked following the acute Q fever episode, only to rise again after 18 months. This increase in antibody titres was accompanied by aspecific complaints of fatigue and a newly diagnosed cardiac murmur. According to the Dutch guidelines, this was a diagnosis of probable chronic Q fever [13], and the patient received antibiotic treatment as considered appropriate.

Interestingly, we observed a normal antibody response in the acute phase of the Q fever infection under anti-TNFα therapy. This is in line with previous studies which have shown that anti-TNFα therapy does not prevent serologic responses to influenza vaccination [14,15], although titres may be somewhat lower. Clearly, in our case, this normal antibody response did not prevent the development of persistent *C. burnetii* infection, as complete clearance might depend more on cellular immune responses.

Indeed, to constrain intracellular *C. burnetii* infections, a cellular immune response is crucial and TNFα is a key cytokine in this response. In-vitro studies showed that TNFα mediates interferon-gamma induced intracellular killing of *C. burnetii* in monocytes through apoptosis [16]. TNF knock-out mice infected with *C. burnetii* develop early bactaeremia and severe heart lesions [17]. Infection risk due to decreased cellular immunity in anti-TNFα treated patients, has been shown for other intracellular infections, most notably *Mycobacterium tuberculosis*, but also *Listeria monocytogenes* and *Salmonella enterica*, and for herpesviridae [18-21].

Rituximab, an anti-CD20 monoclonal antibody, is used for the treatment of RA patients failing on TNFα blockers. Rituximab depletes circulating CD20-positive B-cells for a period of six to nine months [22]. As a consequence, patients on rituximab therapy have an impaired antibody response to neo-antigens. Existing plasma cells and memory B-cells, which do not express CD20, are not affected by rituximab [23]. During the development from acute to chronic Q fever, there is an ongoing infection with presumably increasing concentrations of *C. burnetii* antigens. Because plasma cells do not express B-cell receptors at their surface, which makes them incapable to respond to alterations in antigen concentrations, we assume that the rise of anti-*C. burnetii* IgG titres in our patient originated from stimulation of memory IgG B-cells by increased concentrations of recall antigens.

This intact response to recall antigens after rituximab has been observed for patients receiving vaccinations [24], but has never been documented after natural infection.

Our results indicate that anti-*C. burnetii* phase I IgG antibody titres can be used as a marker for progression to chronic Q fever and the subsequent response to therapy in patients in whom B-cell depleting therapy is started after initial exposure. However, it is likely that B-cell depleting medication during first contact with neo-antigens of *C. burnetii* will seriously hamper the development of an antibody response and the diagnosis of Q fever based on serological titres.

Conclusions

The use of anti-TNFα agents for RA in the acute phase of Q fever does not seem to impede the *C. burnetii*-specific serological response. However, in the presented case, an intact humoral response did not prevent progression to probable chronic *C. burnetii* infection, most likely because essential cellular immune responses were suppressed in the acute phase of the infection. Even though anti-B-cell therapy with rituximab was started after the acute Q fever episode, an increase in anti-*C. burnetii* phase I antibodies was observed, supporting the notion that *C. burnetii* specific CD20-negative memory B-cells are responsible for this rise in antibody titres.

Consent

Written informed consent was obtained from the patient for publication of this Case report.

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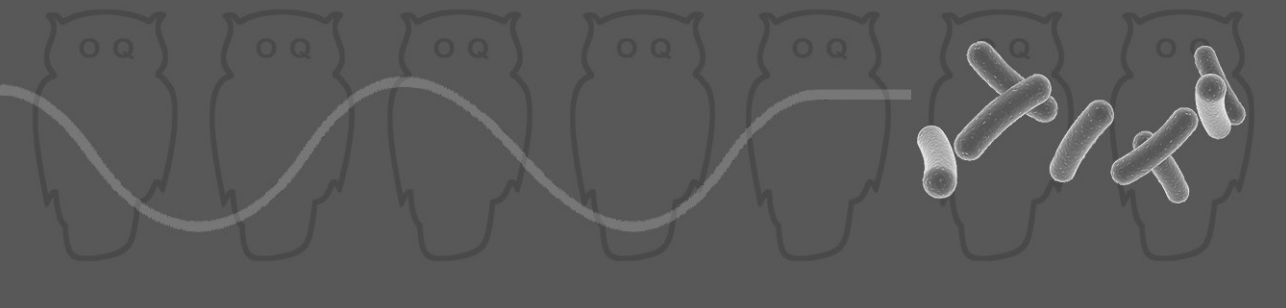
IV

Prevention of Q fever by vaccination against *C. burnetii*



12

Adverse events and association with age, sex and immunological parameters of Q fever vaccination in patients at risk for chronic Q fever in the Netherlands 2011

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Abstract

Background: Following a large Q fever outbreak in the Netherlands, patients at risk for chronic Q fever received a whole-cell Q fever vaccine. Sensitized people were excluded based on pre-vaccination screening with skin test (ST) and serology. An investigational IFN-γ-production assay was added. No previous experience existed for Q fever vaccination in this patient risk-group with predefined cardiac valvular anomalies or aortic aneurysm/prosthesis and many co-morbidities. We studied the adverse events (AE) and their association with patient characteristics and immunological parameters.

Methods: AE registration covered the week after skin test and 90 days following vaccination, with the use of diaries, interviews and spontaneous reports. Serious (S)AE were assessed immediately to ensure safety. We coded AE according to reported severity. Univariate and multivariate analysis addressed associations.

Results: Pre-vaccination screening led to exclusion of 182 patients with positive serology and 207 patients with positive skin test-reading. The skin test did not lead to any causally related SAE. Subsequent vaccination of 1370 patients did not reveal unexpected AE; however, 80% of vaccinees reported local AE (in 26% of these pronounced or extensive). The two causally related SAE (0.1%) both concerned a persistent subcutaneous injection site mass. AE were more frequent in women, younger patients, and those without immunosuppressive co-morbidity/medication. The occurrence of local AE after skin test was associated with pre-vaccination positive serology and high IFN-γ production. This was also true for local AE following vaccination, with a strong association with local AE after skin test as well. The proportion of vaccinees with positive serology and positive IFN-γ values 6 months after vaccination was higher in those with local AE after skin test or after vaccination (non-significant, probably due to small numbers).

Conclusion: Q fever vaccination was safe but reactogenic in this high-risk patient-group. Rates of local AE were higher in women, younger age groups and in those with positive immunological parameters. Vaccinees with local AE after skin test or after vaccination appear to have more pronounced post-vaccination immune responses.

Introduction

Human acute Q fever infection, caused by *Coxiella burnetii*, is asymptomatic in over 50% of cases. In symptomatic cases, clinical presentation ranges from self-limiting febrile illness to pneumonia or hepatitis for which prompt treatment with antibiotics shortens duration [1,2]. Chronic Q fever develops in about 5% of patients, often resulting in endocarditis and vascular infection [3]. In contrast to acute infection, chronic Q fever has high lethality if untreated. Long-term antibiotic therapy, and often surgical intervention, are necessary [4].

In spring 2007, an attentive rural family general practitioner (GP) noticed several cases of atypical community-acquired pneumonia in young adults [5,6]. This proved to be one of the first signals of the largest Q fever outbreak ever [7]. Nearby goat farms were the sources of human infections. Retrospectively, this livestock epidemic started in 2005, following years of intensive goat farming [8]. From 2007 to 2011, 4107 acute Q fever patients were reported, with peak incidence in 2009. Numbers of infected people are estimated to have been ten times as high [9]. Numbers of chronic Q fever increased thereafter, amounting to over 200 cases identified until 2011 with a 13% lethality [10,11]. From 2009 onwards, consecutive measures to curtail the livestock epidemic included mandatory vaccination and culling of carrying animals on infected farms [8]. Some preventive measures, like avoiding human proximity to infected farms, were not feasible due to the dense population.

Several risk-groups for chronic Q fever have been identified, notably those with structural cardiac (valvular) anomalies, prosthetic valves or aortic aneurysms/prostheses [12,13]. The value of antibiotic prophylaxis after initial infection for the prevention of chronic Q fever in these patients has been debated [14,15]. An effective Q fever vaccine is registered in Australia, where – mostly healthy young – people at occupational risk are vaccinated [16].

In 2010, the Netherlands Health Council (HC) advised to vaccinate specific chronic Q fever risk-groups, focusing on the high-incidence area in south-eastern Netherlands [17]. A vaccination campaign was conducted between January and April 2011, with some catch-up vaccinations in June [18]. A centralized approach to screening and vaccination was chosen as the vaccine was not registered in the Netherlands and experience with this vaccine and the required skin test in this specific patient-group was lacking. Pre-vaccination screening was considered essential because previous *C. burnetii* infection is assumed to increase the risk of adverse events (AE) to this whole-cell vaccine [19]. If outcomes of serology or skin test were positive, no vaccination was given [20]. The skin test, however, by intradermal deposition of killed bacterial suspension, may provoke a local reaction. AE following skin test and vaccination were monitored intensively, with follow-up

for 90 days. An investigational whole-blood interferon-gamma (IFN-γ) production assay, measuring cell-mediated immunity in vitro, was added to the screening [21,22]. While its outcome was irrelevant for the decision to vaccinate, it gave an opportunity to analyze AE in vaccinees with possibly pre-existing cell-mediated immunity.

This article describes vaccinees' characteristics, pre-vaccination screening outcomes, and reported AE after skin test and after vaccination in this specific patient-group. We also analyzed the association between local AE after skin test or vaccination, patient characteristics and pre- and post-vaccination immunological parameters [23].

Patients and methods

Target population

Q fever vaccination targeted specific risk conditions, based on the HC advice, with defined exclusion criteria (Box 1). Efforts to reach target populations were most intensive in the high-incidence area. GPs identified and referred patients [18].

Screening and vaccination

Intake and screening took place at a municipal health centre in the high-incidence area, where eligible patients came for screening and subsequent vaccination.

Serology

Antibodies against *C. burnetii* (IgG against phase 1 and phase 2) were measured in 1:32 serum dilution, as described [21]. If positive, exact IgG and IgM titres were determined. Seropositive patients were informed and ST-reading was cancelled. GPs received the results, with interpretation and advice for referral if indicated. Weak IgG signals at 1:32 – 'equivocal' and possibly false positive – did not result in exclusion. Available six months' post-vaccination samples were measured in the same way [22].

Skin test

Trained professionals injected 0.1 mL diluted homologous skin test antigen intradermally in the forearm, as instructed (Q-vax® skin test, CSL, Victoria, Australia) [19]. Skin test (ST)-reading at day 7 included measurement – in millimetres – of redness, swelling and induration. Any induration or swelling with substantial redness – considered positive – led to exclusion. Minimal swelling or local redness alone did not preclude vaccination.

Box 1 Inclusion and exclusion conditions for Q fever vaccination, and coding criteria for adverse events following Q fever skin test and vaccination

Inclusion conditions:

1- history of bacterial endocarditis, other infections of the heart excluded
2- heart valve disease or prosthesis, regardless of type
3- aortic aneurysm or prosthesis, carotid stents excluded
4- congenital heart anomalies, including coarctatio aortae, excluding spontaneous closure of VSD and ductus arteriosus Botalli

Exclusion conditions:

1- age under 15 yrs., pregnancy, incapacity to fulfil requirements, too late referral, severe immunodeficiency
2- history of Q fever infection, positive skin-test or serology, hypersensitivity for vaccine components

Coding criteria adverse events:

a- local redness, swelling	Vaccination:	Skintest:
	1- < 2.5 cm, mild	1 - < 5 mm
	2- 2.5 - < 7.5 cm, moderate	2 - 5 - < 10 mm
	3- 7.5 - < 15cm, pronounced	3 - 10 - < 15 mm
	4- ≥ 15 cm, extensive	4 - ≥ 15 mm
b- local pain	1- pain to the touch, no obstruction of use	
	2- pain on movement, some interference with normal activity	
	3- considerable pain in rest, obstruction of use	
c- fever	1- 37.5 - < 38.5°C, mild	
	2- 38.5 - < 39.5°C, moderate	
	3- 39.5 - < 40.5°C, high	
	4- ≥ 40.5°C, extreme	
d- other systemic events fatigue, headache, chills, gastrointestinal, symptoms, muscle pain, joint pain, etc.	1- easy to endure, minimal discomfort, no impediment	
	2- hindrance and some interference with normal activity	
	3- considerable impediment of normal functioning, need for treatment	
e- duration	1- start and resolve within 3 days after vaccination, early	
	2- start within 3 days and resolve within 6 days after vaccination, long	
	3- start and resolve day 4-6 after vaccination, late	
	4- continuation after day 6, extended	

C. burnetii IFN-γ production-assay

In vitro IFN-γ production after *C. burnetii* stimulation, measured in a whole-blood assay [21], was expressed as continuous variable with cut-off at 32 pg/mL, based on previous findings [21]. Outcome did not affect the decision to vaccinate. Available post-vaccination samples were measured likewise [22].

Vaccination

After negative skin test and serology, vaccination with Q-vax® vaccine (CSL, Victoria, Australia) was performed subcutaneously in the upper arm. This whole-cell vaccine contains formaldehyde-inactivated phase 1 Henzerling strain (25 μg/dose) and thiomersal (0.01% w/v) [19].

Reporting and registration of adverse events

Safety surveillance, covering the week after skin test and 90 days post-vaccination, used diaries, interviews and spontaneous reports. Screened patients should bring the diary with registered AE to ST-reading or, in case of cancellation (mostly related to positive serology), return it by mail. Vaccinees received two additional diaries. The first, for day 0–28 post-vaccination, asked for detailed descriptions of local and systemic AE in the first week, with measurements and experienced severity. Open questions covered the consecutive three weeks. The second diary (month 2–3 post-vaccination), asked questions about illness, medication and doctor's visits, and was returned after 90 days.

Three telephone interviews, after day 7–28–90 respectively, focused on AE, hospital visits and medication, with reminders to return the diaries. Additionally, patients and GPs were advised to report AE. Serious AE (SAE) [24] required expedited reporting to the Health Care Inspectorate, the National Institute for Public Health and Environment and the Netherlands Pharmacovigilance Centre-Lareb, and were immediately assessed in order to enhance the safety of the campaign.

Data analysis

All AE data were entered in a database including indication, comorbidities, medication, and screening/vaccination details. We coded local and systemic AE according to severity, based on the highest scores for size and pain combined (local AE) or on the highest reported grade (systemic AE) (Box 1) [25–27]. We assessed the association with patients' characteristics, pre-vaccination/post-vaccination test outcomes. We used Excel and OpenEpi [28] to compute graphs and to perform statistical analysis. 95% confidence intervals (CI) for proportions and their differences were calculated and Odds ratios (OR) with CI. Multivariate logistic regression in *R* was used to calculate adjusted OR [29].

Results

Description of the population

In total, 1786 patients (out of 2741 referred) were screened (1118 male; 63%) [18]. Their median age was 67 (range 14–92), similar for both sexes, with 1231 (69%) patients being 60 years and over (male 72%, female 64%). Fig. 1 shows the age distribution of the population and its sub-groups in more detail. Reported Q fever risk-conditions were valvular disease (1273; 73%), aortic aneurysm/prosthesis (375; 22%), congenital cardiac anomalies (148; 8%) and prior endocarditis (56; 3%), including multiple risk conditions (109; 6%). For 43 (2%) the risk condition remained unclear. Possible immunosuppression included patients on systemic corticosteroids (45; 2.5%), other immune-modulating drugs (40; 2.2%), with diabetes (198; 11%) and/or current cancer (13; 0.7%). Vitamin K-antagonist medication (665; 39%) indicated increased bleeding risk.

Pre-vaccination screening

Screening led to exclusion of 389 patients (22%) for positive serology (182) or positive ST-reading (207). Another 27 (1.5%) were excluded for other reasons, viz. (perceived) contra-indications, benefit-risk imbalance or intercurrent illness. More men than women had positive screening-tests (25% vs 17%; OR 1.6, CI 1.3–2.1). All together 1370 patients received vaccinations; five of whom despite positive serology, due to miscommunication.

Adverse events following skin test

In total, 1602 (90%) patients supplied information on their experiences in the week following skin test, with higher response for women than for men (93% vs 88%). The response-rate was 97% in vaccinees (1323), and 67% (279) in non-vaccinees, due to cancellation of ST-reading because of positive serology or illness. Vaccinees and non-vaccinees reported local reactions to skin test (ST-locAE) in 39% and 50%, with grade 3–4 AE in 22% and 29%, respectively. Women had higher rates of AE after skin test than men (Table 1). Most of these ST-locAE were early with short duration (63%; 71% in vaccinees, 33% in non-vaccinees). Not surprisingly, late or prolonged local reactions correlated with positive ST-reading (data not shown). Systemic AE after skin test (ST-systAE) were reported in 21% and 22% of vaccinees and non-vaccinees, with grade 3–4 in 2.2% and 2.6% respectively. ST-systAE were mostly non-specific, age-related and/or (aggravated) preexisting complaints. Fever was reported by 3.8%, distributed randomly over the week after skin test. All eight reported SAE, in seven patients, were considered unrelated to the skin test.

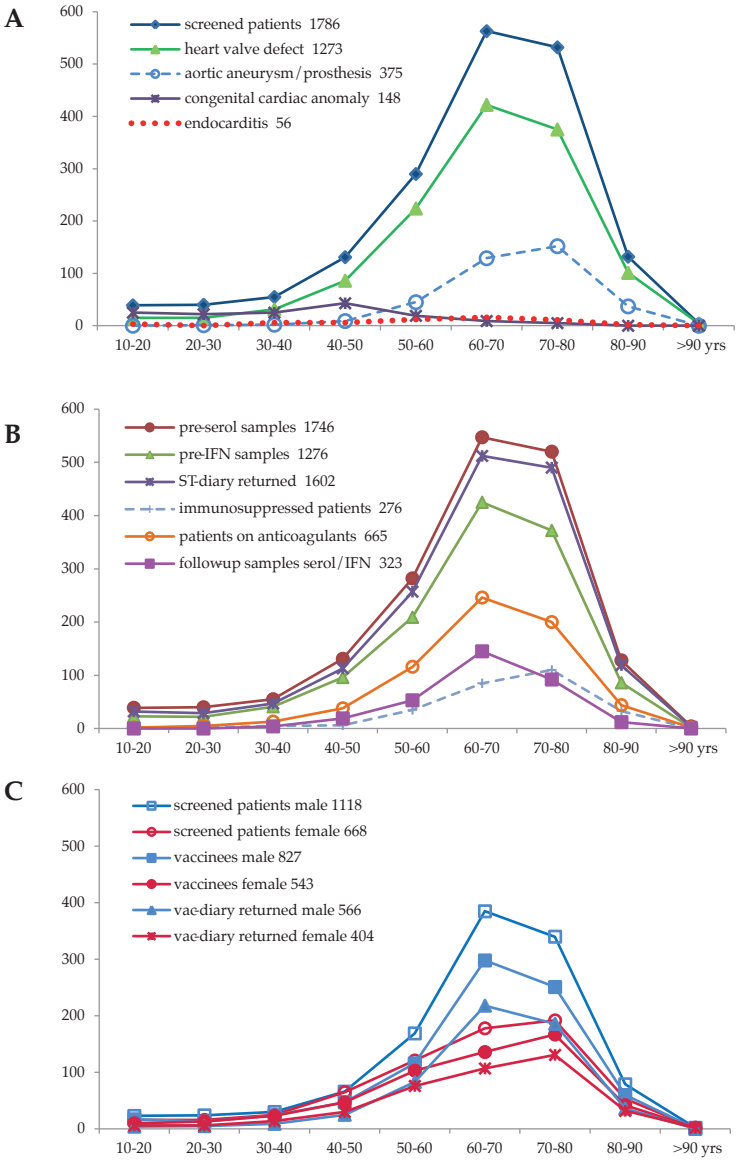

Figure 1. The age distribution of the population with its sub-groups. Age distribution of (A) the screened patients, according to underlying risk conditions; (B) available pre-vaccination blood samples, returned skin test diary, immunosuppressive conditions (including diabetes, malignancy, systemic corticosteroids or other immunosuppressive drugs), anticoagulants and available post-vaccination follow-up samples 6 months after vaccination; (C) the screened and vaccinated patients, and returned vaccination diary, separately shown for male and female sex.

Adverse events following vaccination

Seventy-one percent (970) of the 1370 vaccinees returned diaries, with again higher response-rates for women (74% vs 68%). Based on diary information, local reactions after vaccination (VaclocAE) occurred in 80% (775), of which 21% (208) grade 3–4. Overall, 11% lasted beyond day 6 with mild intensity. Systemic AE after vaccination (Vac-systAE) occurred in 43% (419), including 5.5% (53) grade 3–4. Vaccinees reported fever in 9% (91), all mild/moderate. Women had higher rates of AE than men (88% vs 78%; OR 2.2, CI 1.5–3.1), also for the more severe events (Table 1, Fig. 2a). Younger age groups had higher reporting rates than older, for both sexes (Fig. 2b). Immunosuppression showed an inverse relation with VaclocAE, irrespective sex or age group (Fig. 2c). Vaccinees reported bruising at the injection site in 12% (107; male 7%, female 18%) with 10 reporting grade 3–4, irrespective of anticoagulants.

The 400 vaccinees who did not return diaries reported fewer AE, with any Vac-locAE in 31% (71) and any Vac-systAE in 17% (39) (grade 3–4 14% (32) and 2.7% (6), respectively). These data came from telephone interviews (available for 99.2%), spontaneous reports and follow-up.

The five patients who were vaccinated despite positive serology, did not have prominent AE; two of them reported no AE at all, the other three only moderate Vac-locAE.

Altogether, 104 SAE (in 89 vaccinees) were reported, with two considered possibly related. Both were atypical late and prolonged lumpy local reactions. In one, symptoms resolved about 2.5 years after vaccination. In the other, a dimple still exists. Needle aspiration and ultrasound excluded abscesses. In either case, too deep deposition of the vaccine above the tendon insertion area of the *Musculus deltoideus* may have contributed. The 102 non-related SAE were pre-planned or elective hospital interventions for pre-existing conditions, intercurrent infections and exacerbations of underlying disease or comorbidity.

Association between local AE and pre-vaccination or post-vaccination immunological parameters

ST-locAE and Vac-locAE were tested for association with immunological parameters separately in univariate analysis. We compared proportions and calculated OR (Table 2). Since local AE were associated with sex, age and immuno-suppression, we performed multivariate analysis as well.

ST-locAE was associated with pre-vaccination positive serology and high IFN-γ values and with positive ST-reading at day 7. For vaccination, positive skin test or serology were exclusion criteria; hence association of these positive outcomes and Vac-locAE could not be tested. However, the IFN-γ assay did not influence the decision to vaccinate. Therefore, association with Vac-locAE could be analyzed for

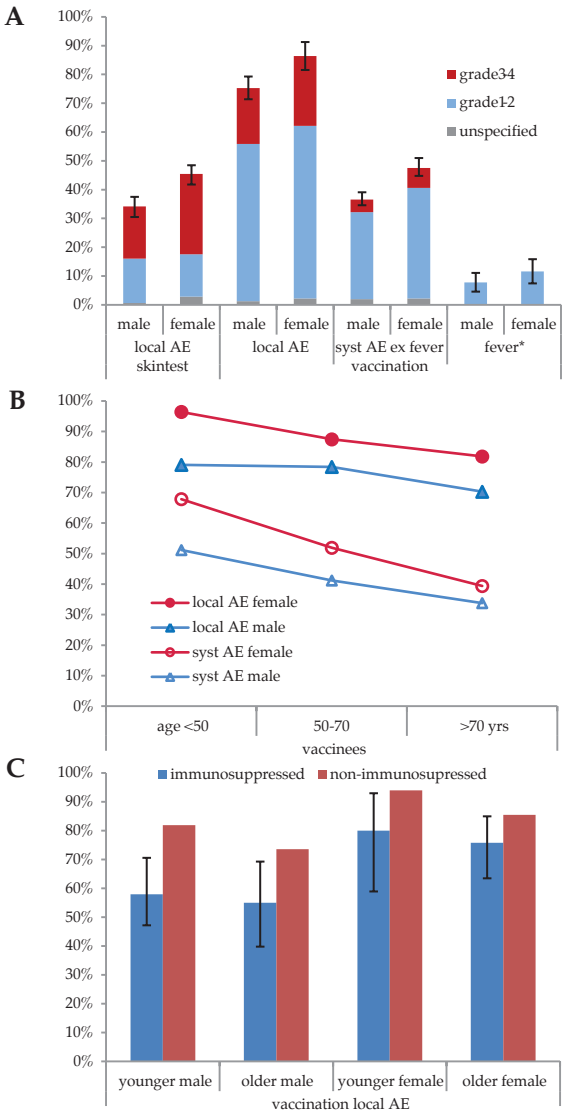

Figure 2. Reported local and systemic AE after skin test and after vaccination, according to sex, age and immunosuppression. (A) Proportion of local and systemic AE after skin test and vaccination according to sex, and graded for severity, with 95% CI. Grade 3–4 after skin test include local AE > 10 mm, while grade 3–4 after vaccination include local AE > 7.5 cm (see also Box 1). Fever after vaccination is shown separately; (B) proportion of local and systemic AEFI according to age-group and sex; (C) proportion of local AEFI in immunosuppressed patients (including diabetes, malignancy, systemic corticosteroids or other immunosuppressive drugs) according to sex and age-group, with 95% CI. Younger male, age < 70 years and younger female, age < 60 years.

Table 1 Local and systemic AE after skin test and vaccination, according to sex. Proportions are based on the number of patients that returned the diary containing data on adverse events after skin test or vaccination

	Adverse events after ST		Adverse events after vaccination				
	Local AE		Local AE		Systemic AE		All AE
	Any[a]	Grade 3-4[b]	Any[a]	Grade 3-4[b]	Any[a]	Grade 3-4[c]	
All; *n*(%)	649 (41)	379 (24)	775 (80)	208 (21)	419 (43)	53 (5.5)	798 (82)
Male; *n*(%)	347 (35)	191 (19)	426 (75)	110 (19)	221 (39)	25 (4.4)	441 (78)
(% CI)	(32.4–38.4)	(17.1–22.1)	(71.6–78.6)	(16–4-22.9)	(35.1–43.1)	(3.0–6.4)	(74.3–81.1)
Female; *n*(%)	302 (49)	188 (30)	349 (86)	98 (24)	198 (49)	28 (6.9)	357 (88)
(% CI)	(44.6–52.4)	(26.8–34.0)	(82.7–89.4)	(20.3–28.7)	(44.2–53.9)	(4.8–9.8)	(84.9–91.1)
OR [F vs M] (CI)	1.7 (1.4–2.1)	1.8 (1.4–2.3)	2.1 (1.5–3.0)	1.3 (1.0–1.8)	1.5 (1.2–1.9)	1.6 (0.9–2.8)	2.2 (1.5–3.1)

Diaries after skin test returned by 1602/1786 (90%) patients (981 male, 621 female). Diaries after vaccination available for 970/1370 (71%) vaccinees (566 male, 404 female).

Abbreviations: AE, adverse events; ST, skin-test; CI, 95% confidence interval; OR, Odds ratio.

[a] Any AE grade 1–4 (defined in Box 1).

[b] Local AE grade 3–4, based on maximum reported severity for redness, swelling and pain (criteria Box 1).

[c] Systemic AE grade 3–4, based on maximum reported grade for any systemic AE, including fever (criteria Box 1).

Table 2 Proportions and Odds ratios for local AE after skin-test and vaccination related to sex, age, pre-vaccination test outcomes and immunosuppression

Skin-test	Univariate association				Multivariate analysis[g]	
	Proportion of local AE		OR (CI) for local AE		aOR (CI) for local AE	
	any[a]	grade 3-4[b]	any	grade 3-4	any	grade 3-4
Sex						
male	35.4	19.5				
female	48.7	30.3	1.7 (1-4-2.1)	1.8 (1.4-2.3)	1.9 (1.5-2.5)	2.5 (1.8-3.4)
Age						
<50 yrs	52.0	35.7	1.4 (1.03-1.9)	1.6 (1.2-2.2)	1.6 (1.04-2.33)	1.9 (1.2-3.0)
50-70 yrs (ref)	43.8	25.6				
>70 yrs	32.4	17.0	0.6 (0.49-0.77)	0.6 (0.46-0.78)	0.69 (0.52-0.92)	0.68 (0.48-0.97)
Pre-vaccination serology[c]						
negative (ref)	38.9	22.4				
equivocal	43.2	24.6	1.2 (0.81-1.7)	1.1 (0.79-1.5)	1.2 (0.76-1.9)	1.4 (0.82-2.5)
low positive	60.0	33.3	2.4 (1.1-5.1)	1.7 (0.77-3.7)	3.6 (1.3-10.0)	3.6 (1.1-12.0)
high positive	61.0	45.8	2.5 (1.4-4.2)	2.9 (1.7-5.0)	2.2 (1.1-4.3)	2.8 (1.3-6.0)
any positive(ref neg+equiv)	50.7	31.8	1.6 (1.2-2.2)	1.6 (1.2-2.2)		
Pre-vaccination IFN-γ assay[d]						
negative (ref)	39.7	21.6				
low positive	40.0	24.8	1.01 (0.69-1.5)	1.2 (0.77-1.8)	1.0 (0.66-1.5)	1.2 (0.73-2.0)
high positive	53.7	33.1	1.8 (1.2-2.5)	1.8 (1.2-2.7)	1.5 (0.98-2.3)	1.8 (1.1-3.0)
any positive	47.1	29.1	1.4 (1.02-1.8)	1.5 (1.1-2.0)		
ST-reading[e]						
negative	38.9	22.2				
positive	48.2	30.4	1.5 (1.1-1.9)	1.5 (1.1-2.0)	nd[h]	nd[h]
Immunosuppression[f]						
no	41.6	25.0				
yes	33.9	15.9	0.72 (0.54-0.96)	0.57 (0.39-0.81)	0.77 (0.54-1.1)	0.73 (0.46-1.15)

Vaccination	Proportion of local AE		OR (CI) for local AE		aOR (CI) for local AE	
	any[a]	grade 3-4[b]	any	grade 3-4	any	grade 3-4
Sex						
male	75.1	19.4				
female	86.1	24.3	2.1 (1.5-2.9)	1.3 (0.98-1.6)	1.9 (1.2-3.1)	2.1 (1.1-3.8)
Age						
<50 yrs	88.9	30.3	1.8 (0.93-3.6)	1.3 (0.83-2.2)	1.5 (0.53-4.2)	0.96 (0.26-3.5)
50-70 yrs (ref)	81.8	24.4				
>70 yrs	74.7	15.5	0.66 (0.47-0.91)	0.57 (0.40-0.80)	0.72 (0.37-1.1)	0.50 (0.27-0.93)
Pre-vaccination serology[c]						
negative	79.2	21.2				
equivocal	84.5	25.9	1.4 (0.71-3.1)	1.3 (0.68-2.4)	1.9 (0.68-5.4)	3.4 (0.92-12.6)
Pre-vaccination IFN-γ assay[d]						
negative (ref)	79.4	21.3				
low positive	85.3	27.9	1.5 (0.77-3.2)	1.4 (0.81-2.5)	1.2 (0.57-2.6)	0.76 (0.28-2.1)
high positive	87.9	33.3	1.9 (0.70-6.4)	1.8 (0.84-3.9)	1.9 (0.58-6.1)	3.1 (0.77-12.6)
any positive	86.1	29.7	1.6 (0.90-3.0)	1.6 (0.96-2.5)		
ST local AE						
none (ref)	70.3	15.4				
grade 1-2	90.7	25.2	4.2 (2.4-7.5)	1.8 (1.2-2.8)	5.1 (2.4-10.7)	7.7 (3.2-18.5)
grade 3-4	95.6	33.8	9.2 (4.9-18.8)	2.8 (2.0-4.0)	7.1 (3.1-16.1)	11.7 (4.5-30.4)
any	93.6	30.2	6.2 (4.0-9.8)	2.4 (1.7-3.3)		
Immunosuppression[f]						
no	82.0	22.9				
yes	65.6	12.9	0.42 (0.29-0.61)	0.50 (0.30-0.80)	0.40 (0.24-0.65)	0.27 (0.13-0.59)

Abbreviations: ST, skin-test; AE, adverse event; gr 3-4, severity grade 3 or 4; IFN-γ, interferon-γ; OR, odds ratio; aOR, adjusted Odds Ratio; nd, not done.
[a] Any local AE includes grade 1-4, as defined in Box 1.
[b] Local AE grade 3-4 based on maximum reported severity for redness, swelling and pain (criteria Box 1).
[c] Negative is <1:32; Equivocal, weakly positive IgG at 1:32; Low positive, ≥ 1:32 and <1:128; High positive, ≥1:128.
[d] Negative <32 pg/mL; Low positive ≥ 32 pg/mL and <100 pg/mL; High positive ≥ 100 pg/mL.
[e] Positive when any induration or swelling with substantial redness at ST-reading at day-7.
[f] Immunosuppression due to co-morbidities or medication: including diabetes, malignancy, use of systemic corticosteroids or other immunosuppressive drugs.
[g] Multivariate logistic regression to calculate aOR with CI.
[h] ST-reading was not included in the multivariate logistic regression model.

Table 3 Proportions and Odds ratios for post-vaccination immune responses in vaccinees, related to sex, age, pre-vaccination tests, local AE after skin-test or vaccination and immunosuppression

Post-vaccination serology	Univariate association				Multivariate analysis[g]	
	Proportion positive serology		OR (CI) for positive serology		aOR (CI) for positive serology	
	Positive[a]	High positive[b]	Positive	High positive	Positive	High positive
Sex						
male	43.8	21.0				
female	47.6	21.4	1.2 (0.69-2.9)	1.0 (0.53-1.9)	1.8 (0.97-3.4)	2.0 (0.86-4.5)
Age						
<50 yrs	55.6	33.3	1.6 (0.60-4.5)	1.9 (0.63-5.5)	1.8 (0.52-6.3)	2.1 (0.49-8.8)
50-70 yrs (ref)	43.5	20.5				
>70 yrs	45.7	19.8	1.1 (0.63-1.9)	0.96 (0.48-1.9)	1.37 (0.71-2.6)	1.2 (0.49-2.8)
Pre-vaccination serology[c]						
negative	42.2	20.1				
equivocal	87.5	37.5	9.5 (2.4-63.0)	2.4 (0.77-6.9)	9.1 (1.9-44.5)	6.1 (0.96-39.4)
ST local AE[d]						
none (ref)	42.6	16.3				
grade 1-2	42.9	18.4	1.01 (0.52-2.0)	1.2 (0.47-2.7)	0.93 (0.41-2.1)	1.2 (0.40-3.4)
grade 3-4	53.1	32.8	1.5 (0.84-2.8)	2.5 (1.2-5.0)	1.1 (0.52-2.3)	1.6 (0.66-4.0)
any	48.7	26.5	1.3 (0.78-2.1)	1.9 (1.00-3.4)		
Vaccination local AE[d]						
none (ref)	29.4	8.8				
grade 1-2	46.6	22.9	2.1 (0.93-4.9)	3.1 (0.95-13.4)	1.4 (0.58-3.6)	1.7 (0.41-6.8)
grade 3-4	54.0	20.1	2.8 (1.1-7.3)	2.6 (0.68-12.4)	2.0 (0.70-5.5)	1.7 (0.34-8.0)
any	48.6	22.1	2.3 (1.04-5.2)	2.9 (0.93-12.6)		
Immunosuppression[e]						
no	47.4	22.7				
yes	31.0	11.9	0.50 (0.24-1.00)	0.46 (0.15-1.2)	0.60 (0.25-1.4)	0.41 (0.10-1.6)

Post-vaccination IFN-γ assay	Proportion positive IFN-γ assay		OR (CI) for positive IFN-γ assay		aOR (CI) for positive IFN-γ assay	
	Positive[a]	High positive[b]	Positive	High positive	Positive	High positive
Sex						
male	64.8	38.1				
female	59.8	24.4	0.81 (0.47-1.4)	0.53 (0.29-0.94)	0.97 (0.49-1.91)	0.67 (0.28-1.6)
Age						
<50 yrs	70.6	52.9	1.2 (0.41-4.0)	2.3 (0.84-6.7)	0.99 (0.26-3.9)	1.7 (0.35-8.3)
50-70 yrs (ref)	66.5	32.3				
>70 yrs	55.0	32.5	0.62 (0.36-1.1)	1.01 (0.56-1.8)	0.69 (0.35-1.38)	0.86 (0.38-2.0)
Pre-vaccination IFN-γ assay[f]						
negative (ref)	59.7	27.2				
low positive	87.5	66.7	4.7 (1.5-20.4)	5.3 (2.2-13.8)	3.1 (0.80-12.1)	5.4 (1.3-22.4)
high positive	84.6	76.9	4.0 (0.89-25.1)	8.8 (2.4-41.1)	2.9 (0.56-14.9)	5.4 (0.96-29.6)
any positive	86.4	70.3	4.3 (1.7-12.9)	6.3 (2.9-14.1)		
ST local AE[d]						
none (ref)	62.9	32.1				
grade 1-2	67.3	38.8	1.2 (0.61-2.5)	1.3 (0.67-2.6)	1.2 (0.51-2.9)	1.3 (0.47-3.7)
grade 3-4	61.9	34.9	0.96 (0.52-1.8)	1.1 (0.60-2.1)	0.78 (0.36-1.7)	0.79 (0.31-2.1)
any	64.3	36.6	1.1 (0.63-1.8)	1.2 (0.72-2.1)		
Vaccination local AE[d]						
none (ref)	55.9	32.4				
grade 1-2	59.2	28.5	1.1 (0.53-2.5)	0.83 (0.37-1.9)	0.76 (0.30-1.9)	0.57 (0.19-1.7)
grade 3-4	70.0	48.0	1.8 (0.73-4.6)	1.9 (0.77-4.9)	1.1 (0.37-3.2)	1.3 (0.37-4.2)
any	62.2	33.9	1.3 (0.61-2.7)	1.1 (0.49-2.4)		
Immunosuppression[e]						
no	63.6	34.0				
yes	54.8	28.6	0.69 (0.35-1.4)	0.78 (0.38-1.6)	0.59 (0.25-1.4)	0.58 (0.20-1.7)

Post-vaccination samples were available for 260 vaccinees.
Abbreviations: ST, skin-test; AE, adverse event; IFN-γ, interferon-γ; CI, 95% Confidence Interval; OR, odds ratio; aOR, adjusted Odds Ratio.
[a] Serology IgG phase 1 or 2 ≥1:32 or IFN-γ assay ≥32 pg/mL, respectively.
[b] Serology IgG phase 1 or 2 ≥1:128 or IFN-γ assay ≥100 pg/mL, respectively.
[c] Serology is negative when IgG <1:32; equivocal, weakly positive IgG at 1:32.
[d] Local AE grades are defined in Box 1. Local AE grade 3-4 is based on maximum reported severity for redness, swelling and pain (criteria Box 1).
[e] Immunosuppression due to co-morbidities or medication: including diabetes, malignancy, use of systemic corticosteroids or other immunosuppressive drugs.
[f] IFN-γ assay is negative when <32 pg/mL; low positive, ≥32 pg/mL and <100 pg/mL; high positive, ≥100 pg/mL.
[g] Multivariate logistic regression was used to calculate aOR with CI.

all values. Proportions of Vac-locAE increased with higher values of IFN-γ production, but OR were non-significant. ST-locAE was strongly associated with Vac-locAE (point estimates of adjusted OR ranging from 5.1 to 11.7).

Association between Local AE and post-vaccination immunological parameters, i.e. serology and IFN-γ production were tested univariately as well (Table 3). For both these immunological parameters, OR suggested association with Vac-locAE, but results were statistically non-significant, possibly because of small numbers. In multivariate analysis adjusted for sex, age, immunosuppression and pre-vaccination immunological parameters, OR roughly confirmed these associations.

Discussion

This unprecedented Q fever vaccination campaign, in the after-math of the Q fever outbreak in the Netherlands, targeted a group with defined high-risk conditions for chronic Q fever. The need for pre-vaccination screening and the non-licensed vaccine in Europe were the main reasons for choosing a centralized approach. The much higher than expected referral rate affected implementation. The lack of prevalence data for risk conditions precluded also coverage estimation (manuscript in preparation). Compared with previous Australian target-groups, (i.e. healthy abattoir workers [30]) our target population involved more women, older age-groups, and many co-morbidities/medications. Included risk-groups for vaccination fit the recently described risk factors for chronic Q fever in the Netherlands: valvular surgery (OR 43.6), aneurysm (OR 25.9) and vascular prosthesis (OR 26.8) [13]. Pregnant women, also considered at risk for chronic Q fever [31–33], were excluded in this vaccination-campaign because of safety concerns.

The campaign aimed at individual protection through vaccination of patients at high risk for chronic Q fever and was not designed as a study. This resulted in several weaknesses in data collection and reports, like limited validation/supplementation of comorbidities and medication, and cancellation of ST-reading when serology turned out to be positive. The strength of this study, however, is the high response-rate for diaries after skin test and vaccination, and a follow-up of 99.2%. This means that any safety signal should have been picked-up. In addition, we could study the association between AE, patient characteristics and immunological parameters, inclusive of the incorporated IFN-γ production assay.

ST, measuring cell-mediated immunity, is the primary screening test (internationally) and is complemented by serology, reflecting humoral immunity [20,34]. The discordance of positive skin test and absence of antibodies (207; 12%) confirms previous observations [35]. Skin test did not lead to any ST-related SAE. The 3.8% with reported fever may reflect the background rate. Although skin test aims to

provoke late local reactions in sensitized people, it may result also in early inflammatory redness, swelling, pain or bruising, not representing T-cell mediated responses. Vaccinees reported ST-locAE in 39%, which were mostly early, mild/moderate [<10 mm], and short-lived. This illustrates remittance of early ST-locAE, resulting in negative ST-reading at day 7. This seven-day timeframe of ST-reading (as directed [20]) appears based on a study showing 58 people ST-positive after 7–9 days, of whom only 43 after 2 days. In 30 others with previous Q fever infection, all were ST-positive after 7–9 days, but only 19 after 2 days [36]. Interestingly, our data show that self-reported ST-locAE within one week strongly predicted occurrence of Vac-locAE, without, however, severe persistent Vac-locAE or systemic hypersensitivity. This association may reflect individual tendency for hyperreaction rather than immunological hypersensitivity.

Intensified surveillance after vaccination revealed no unexpected AE, although higher AE rates than previously published [16,20,30]. Rates, however, are not easily comparable as studies differ in target population and design of surveillance [27]. Here, proportions of reported AE were consistently higher in women than in men, both for milder and more pronounced AE. Few publications about AE differentiate between sexes and age [37–39]. Yet, we have noted such differences in several vaccination programmes for adults and children, depending on age and type of event [40]. Here, in addition to sex, age influenced the occurrence of AE after vaccination, as did immunosuppression by medication or illness.

The vaccinees with returned diaries had high reporting-rates for Vac-locAE (80%), but the 29% vaccinees without diaries had much lower rates (31%) This is not surprising since having AE is an incentive to return the diary. Therefore, these rates reflect worst-case and best-case estimates. The average (71%) may be the more realistic rate.

Fever was a relatively rare symptom (9%), with temperatures only moderately elevated. This may be because elderly people tend to respond less with high temperature to vaccinations or infections [41]. The pre-vaccination IFN-γ production, as measured in the whole-blood assay, showed a positive association with Vac-locAE, but the observed differences did not reach statistic significance. The value of this observation is difficult to interpret, since patients that had any other positive parameter of pre-existing immunity (positive serology or ST) were not vaccinated (except in five cases of miscommunication, as mentioned before). Positive IFN-γ values were overrepresented in non-vaccinees; therefore, the found associations are more likely to be underestimated than overestimated. Missing values for immunosuppression and for ST-locAE were differential as well, overrepresented in the non-vaccinees, who had higher proportions of positive antibody titres and of ST-locAE. This may have introduced a bias, with underestimation of found associations.

We previously published limited immune responses in this old-age group with much co-morbidity, with as many as 23% negative serology and IFN-γ values after six months [21]. However, despite limited serological responses to vaccination, Australian studies have shown high protection in occupationally exposed people [30,42–44]. Our data suggest that local AE after skin test and/or vaccination were associated with post-vaccination immune responses. Either the local reaction stimulates the immune response, or it is a (early) side effect in the process of adaptive immune responses itself.

The safety of Q fever vaccination has been subject of discussion, with severe "hypersensitivity" reaction being a major issue [17]. This is assumed to occur after vaccination in previously sensitized individuals, hence the stringent pre-vaccination screening regimen [34,45]. With time, concerns seem to have increased, without additional support by reliable data. A recent report on passive surveillance of the Australian national Q fever vaccination campaign 2001–2004, showed a very low number of reported AEFI (86 AEFI in 48,986 vaccinees; 0.002%). These included mostly local reactions, with only one SAE [16].

Whether pre-vaccination screening has prevented many serious AEFI in the Dutch campaign remains an unanswered question, as positive skin test or serology were exclusion criteria for vaccination. It may be argued, however, that the stringent screening possibly withheld protection from those with low-grade reactivity or low titres. Whether the prominent role of serology has contributed to justified exclusion, is debatable.

Of note, only 33.3% (11/33) of the vaccinees with pre-vaccination high IFN-γ production, reflecting pre-existing cell-mediated immunity, experienced pronounced or extensive local (transient) AE, but anaphylaxis did not occur. The five people erroneously vaccinated, all with positive serology and in two borderline ST-reading as well, did not have prominent AE. Pre-vaccination equivocal serology was not significantly associated with local AE but, interestingly, showed significant and strong association with higher post-vaccination serological response. Overall, only two reported so-called SAE, with atypical lumpy local reactions, were considered causally related, with no suggestion of abscess or granuloma as previously described [19,26,46,47]. These two patients had negative serology, negative ST-reading, did not report late or extended ST-locAE, and one had negative IFN-γ (for the other no sample was available).

Thus, we conclude that this Q fever vaccination campaign has been safe, with a large proportion of vaccinees reporting local injection site reactions after vaccination (80%). These local reactions, however, were mostly non-severe and of limited duration. We stress the merit of differentiating according to sex, age and other covariates in reporting of AEFI. We dispute the base for the stringent requirement of pre-vaccination testing, which needs further evaluation in experimental setup.

Pre-vaccination screening, which is current practice, hampers appropriate and/or efficient use of the vaccine. Development of an effective and less reactogenic vaccine could make pre-vaccination screening unnecessary at all.

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Supplementary data

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Limited humoral and cellular responses to Q fever vaccination in older adults with risk factors for chronic Q fever

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Abstract

Objectives: In the Netherlands, people at risk for chronic Q fever were vaccinated against *Coxiella burnetii* with the inactivated whole cell vaccine Q-vax. We aimed to measure the immune responses to *C. burnetii* six and twelve months after vaccination in this relevant population.

Methods: In 260 vaccinees, antibody responses were assessed by immunofluorescence assay (IFA), complement fixation test and ELISA. The cellular immune responses were assessed by measuring *C. burnetii*-specific interferon (IFN)-γ production in blood. Serological results of 200 individuals with past Q fever were used for comparison.

Results: At six months, 46% of vaccinees showed low IFA antibody titres and 67% had a positive IFN-γ assay; At twelve months, both were 60%. In contrast, individuals with a past Q fever were seropositive in 99.5% at six and twelve months, with relatively higher IFA titres. Interestingly, vaccinees with positive IFN-γ assay pre-vaccination, showed a higher seroconversion rate than IFN-γ negative vaccinees: 74% vs. 41% ($p < 0.001$).

Conclusions: The immune response after Q-vax vaccination is lower and restricted to a smaller proportion than found after past Q fever and than previously described after vaccination, suggesting decreased vaccine immunogenicity in this high-risk population. A positive IFN-γ assay before vaccination in seronegative vaccinees likely points to pre-existing immunity resulting in boosting by vaccination.

Introduction

Q fever is a zoonosis caused by the intracellular bacterium *Coxiella burnetii*, which has its main reservoir in sheep, goat and cattle.[1] Q fever is endemic in some areas,[2] but may also occur in outbreaks.[3-5] Between 2007 and 2010, the Netherlands faced the largest Q fever outbreak ever reported,[6] with an estimate of more than 40,000 infected individuals [7] and more than 4000 notified human cases of acute Q fever. Infection may remain asymptomatic or non-specific.[8] If symptoms occur, acute Q fever, presenting with fever, pneumonia or hepatitis, rarely causes severe disease.[9] However, months till years after first contact, 1-5% of the infected individuals may develop chronic infection, mostly endocarditis or vascular wall infection,[1] for which longterm antibiotic treatment and often surgery is needed. If left untreated, chronic Q fever has a mortality rate as high as 60%.[10] Factors that predispose to chronic Q fever are pre-existent valvular or vascular disease,[1] conditions that are mainly met in older frail people often suffering from comorbidity. In 2009, measures were taken to prevent new human cases, a.o. mandatory vaccination of dairy goats and sheep and culling of non-immunized pregnant goats and sheep on *C. burnetii* positive farms. In 2010, it was not clear if these veterinary measures were effective enough. Therefore, human vaccination was considered, since that may prevent Q fever infection.[11,12] In June 2010, the Dutch health authorities decided to offer vaccination to all individuals at risk for chronic Q fever living in the area of the epidemic.

The only Q fever vaccine currently available for human use is Q-vax (CSL Biotherapies, Victoria, Australia) an inactivated whole cell vaccine licensed in Australia, which contains the phase I Henzerling strain.[13] Vaccination is routinely performed in Australian workers who are occupationally exposed to *C. burnetii*.[14] As was shown by various studies, vaccination in these mainly young male employees induces humoral and cellular immune responses[15,16] and protects against infection.[17,18] However, no data are available on the immune response to Q-vax in older adults at risk for chronic Q fever.

In the present study, we report the antibody responses to Q-vax, as measured by various techniques, as well as the in vitro C. burnetii-induced interferon (IFN)-γ production at 6 and 12 months after vaccination in 260 vaccinated elderly individuals with risk factors for chronic Q fever.

Material and Methods

Setting

The Dutch Q fever vaccination campaign took place in the spring of 2011 in the Municipal Health Service in 's Hertogenbosch.[19] All vaccine candidates were screened one week before vaccination by serology using an immunofluorescence assay (Focus Diagnostics, Cypress, USA) and skin testing (Q-vax® Skin Test, CSL Biotherapies, Victoria, Australia)[13] according to the manufacturer's instruction and as previously described.[20] Simultaneously, an IFN-γ production assay was performed to assess in vitro cell-mediated immunity to *C. burnetii*.[21] The outcome of this test did not affect the decision to vaccinate. Those individuals with negative outcome in serology and skin tests were vaccinated. One dose of Q-vax® vaccine (lot # 0980-07501) was administered subcutaneously in the upper arm according to the product information.[13] Following measures in the veterinary sector, the epidemic subsided after 2010. Therefore, in the year after vaccination exposure to C. burnetii was unlikely.

Subject enrollment

Following the vaccination campaign, in which 1368 individuals were vaccinated,[20] 260 vaccinees were included in the study. After obtaining written informed consent, blood for microbiological screening was collected approximately 6 and 12 months after vaccination. Information about morbidity and medication was collected in a questionnaire. Control sera were retrieved from a cohort of 371 anonymous individuals, enrolled in a follow-up study after a natural acute Q fever infection in 2009 in the Jeroen Bosch Hospital in 's-Hertogenbosch. Sera taken 6–7 months and 12–13 months after natural infection were eligible. From these, the 200 oldest individuals were evaluated since they matched the vaccinees as close as possible. The study was approved by the Central Committee on Research on Human Subjects (CCMO).

Microbiological screening

Antibody response measurements

Serum samples of the vaccinees were aliquoted and tested for anti-*C. burnetii* phase I and phase II antibodies with three methods available for serological screening: immunofluorescence assay (IFA), complement fixation test (CFT) and enzyme-linked immunosorbent assay (ELISA). Control sera were tested with IFA only.

A commercially available IFA kit (Focus Diagnostics, Cypress, USA) was used according to the manufacturer's instructions. The assay measures IgM and IgG against *C. burnetii* Nine Mile phase I and II antigens. Seropositivity is described for antibody classes separately and overall as any anti-C. burnetii titre ≥1:32.

CFT (Virion/Serion, Würzburg, Germany) was performed, according to the manufacturer's instructions, to measure phase II antibodies. After the procedure steps, a ready-to-use hemolytic system (Virion/Serion) was used. Dilutions with ≤50% of hemolysis were defined as positive. A positive result was defined as having an endpoint dilution of at least 1:10.

With ELISA (Virion/Serion), IgG against phase I and II antigens and IgM against phase II were measured. IgG phase I and IgM phase II were positive when the absorbance was more than 10% above the extinction of the cut-off control. Ambiguous results were added to negative results.

Interferon-gamma production measurement

Heparinized whole blood was aliquoted into 0.5 ml in separate tubes and incubated 24 h at 37 ^{O}C with *C. burnetii* antigens (heat-inactivated *C. burnetii* Nine Mile RSA 493 phase I, [10^7 bacteria/mL]), nil or PHA as previously described.[21] IFN-γ was measured by ELISA (Pelikine compact, Sanquin, Amsterdam, The Netherlands). Net IFN-γ production was expressed as the concentration of IFN-γ in the *C. burnetii* stimulated sample minus that in the negative control sample. The assay was considered positive when net IFN-γ production was ≥32 pg/ mL, based on previous findings.[21]

Statistics

Proportions of positive results were calculated and compared among different tests using McNemar test. Reciprocal antibody titres were used for comparison. Geometric mean antibody titres were calculated and compared using a student *t*-test. A titre <1:32 was assigned 1:16 for calculations. Quantitative IFN-γ production (pg/mL) was expressed as mean (±standard error [SE]) per group. Agreement between detection of antibody response and IFN-γ response was assessed and the kappa (κ) statistic measure was calculated. The proportional Venn diagram was drawn using the online DrawVenn application.[22] Logistic regression was used to assess the association between absent immune response and the main risk factors for immunosuppression.

Results

Subjects' characteristics

A total of 260 vaccinees were studied, with a mean age (±standard deviation (SD)) of 65.1 (±9.3) years. 66.2% of the vaccinees were male. All had negative serological and skin test outcome in pre-vaccination screening. The IFN-γ assay prior to vaccination was negative in 197 (76.0%) vaccinees, positive in 42 (16.0%) and inconclusive in 21 (8.0%). Table 1 shows the characteristics of the study population and the subgroups based on pre-vaccination IFN-γ production.

The mean (±SD) duration between vaccination and blood collection was 6.9 (±0.6) and 11.8 (±0.4) months respectively. Fig. 1 shows the number of vaccinees with blood sampled at six and at twelve months after vaccination and the number of results available for serological and IFN-γ assays.

The control group of 200 individuals with a past natural *C. burnetii* infection had a mean age (±SD) of 57.5 (±10.7) years; 61.5% was male. The serum samples for serological follow-up were obtained between 6-7 months and 12-13 months after the acute Q fever episode.

Antibody response after vaccination

Results of serological assays are shown in Table 2. At six months, results for all serological tests were available of 257 subjects, at twelve months of 227 individuals. Among the three serological tests, measurement of anti-*C. burnetii* IgG antibodies with IFA showed the highest proportion of seropositive individuals: at six months 38.5% IgG against phase I and 34.2% against phase II, while at twelve months this was 51.5% and 33.5%, respectively. IgM antibodies were detected in only a small proportion. Considering any IgG/IgM IFA titre ≥1:32 as a positive outcome, the proportion seropositives was 45.9% at six and 59.9% at twelve months after vaccination. The other serological tests were positive in a significantly smaller number of subjects: CFT in 16.0% ($p < 0.001$) at six and 6.6% ($p < 0.001$) at twelve months, ELISA 24.1% ($p < 0.001$) and 26.9% ($p < 0.001$), respectively.

In the control group of 200 patients with a past natural infection, 99.5% showed anti-*C. burnetii* IgG antibodies in the IFA at both six and twelve months after infection. Comparison of the IFA IgG titres of the vaccinees and the naturally infected individuals showed significantly lower antibody titres for the vaccinees (Fig. 2). This was especially the case for IgG anti-phase II. Six months after vaccination, the geometric mean reciprocal titres of IgG anti-phase I and anti-phase II were 32.7 and 28.2, respectively, compared to 102.9 and 1999.0, six months after natural infection ($p < 0.0001$). Twelve months after vaccination, this was 38.2 and 26.0 compared to 97.7 and 1531.0 ($p < 0.0001$), respectively.

IFN-γ production after vaccination

The IFN-γ production assay, measuring a cell-mediated immune response, provided positive results in 63% (161/255) of vaccinees at six and in 54% (122/227) at twelve months. However, the IFN-γ assay was inconclusive in 13 and 21 subjects at six and twelve months, respectively. When only taking into account the conclusive results of the IFN-γ test, the proportion positive results was 67% (161/242) at six and 60% (123/206) at twelve months.

Table 1 Characteristics of vaccinated subjects followed-up after vaccination.

	All vaccinees (n=260)	Vaccinees with pre-vaccination negative IFN-γ assay, (n=197)	Vaccinees with pre-vaccination positive IFN-γ assay, (n=42)	*P* value (vaccinees with negative vs. vaccinees with positive IFN-γ assay) [e]
Mean age, yrs (±SD) [a]	65.1 (9.3)	65.4 (9.2)	62.9 (8.8)	0.11 [f]
Male	172 (66.2)	130 (66.0)	30 (71.4)	0.59
Use of immunosuppressive drugs[b]	11 (4.2)	9 (4.6)	1 (2.4)	1.00
Medical history:				
Auto-immune disease	13 (5.0)	10 (5.1)	1 (2.4)	0.69
Diabetes mellitus[c]	30 (11.6)	22 (11.2)	4 (9.5)	1.00
COPD / asthma	30 (11.6)	25 (12.7)	3 (7.1)	0.43
Malignancy[d]	7 (2.7)	6 (3.0)	1 (2.4)	1.00

No. (%) indicates subjects with information available for that category.
Abbreviations: SD, standard deviation; COPD, chronic obstructive pulmonary disease; IFN- γ, interferon-gamma.
[a] Age at pre-vaccination screening.
[b] Including prednisone, disease modifying anti-rheumatic drugs (DMARDs), mesalazine, hydroxyurea.
[c] Type 1 or type 2.
[d] Currently or past with current treatment, including hematologic malignancies, excluding basocellular carcinoma.
[e] Fisher's exact test, unless otherwise indicated.
[f] Independent-samples *t* test.

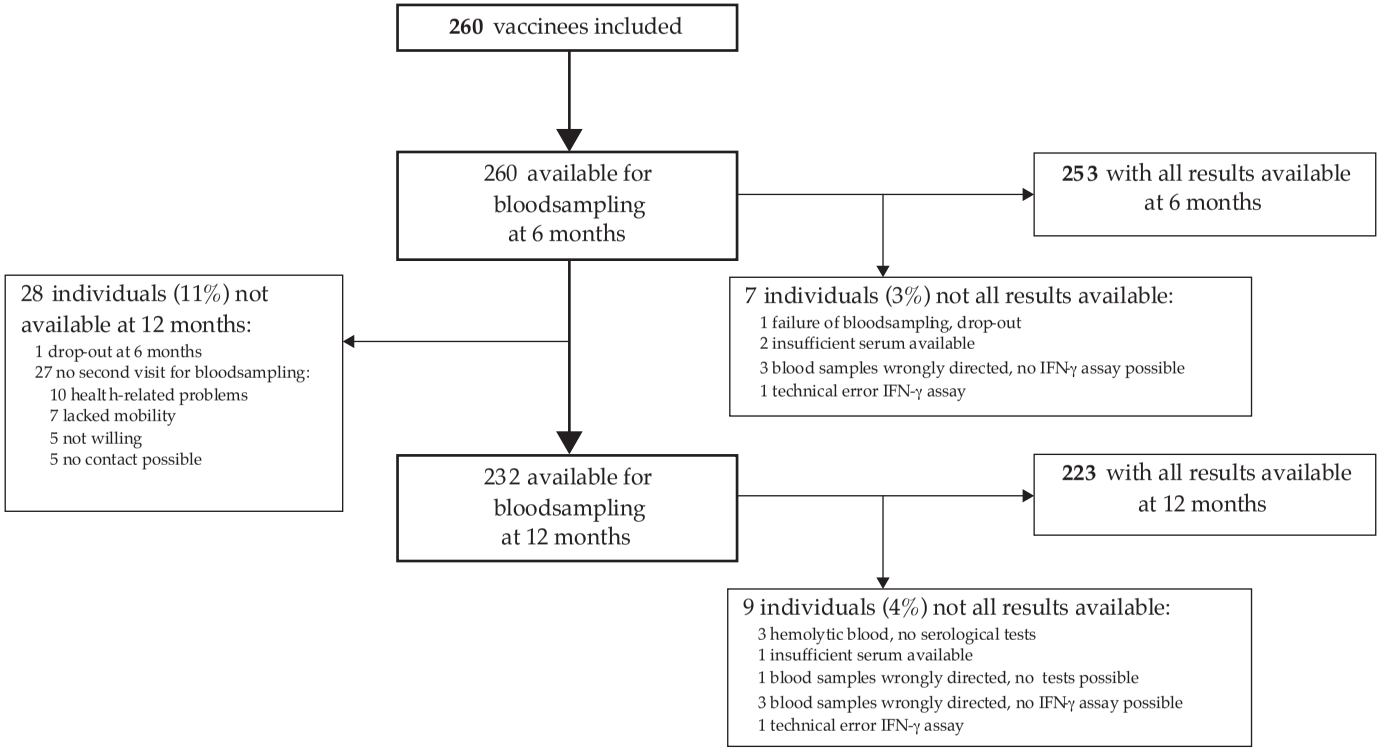

Figure 1. Flow chart of study subjects. Abbreviations: IFN-γ, interferon-gamma

Table 2 Serological assay and IFN-γ assay outcomes of vaccinated subjects 6 and 12 months after vaccination

Assay	6 months after vaccination, No. (%) tested positive [a]	12 months after vaccination, No. (%) tested positive [a]
IFA total [b]	118 (45.9)	136 (59.9)
IFA, IgG phase I	99 (38.5)	117 (51.5)
IFA, IgG phase II	88 (34.2)	76 (33.5)
IFA, IgM phase I	26 (10.1)	25 (11.0)
IFA, IgM phase II	12 (4.7)	8 (3.5)
CFT, phase II	41 (16.0)	15 (6.6)
ELISA total [b]	62 (24.1)	61 (26.9)
ELISA IgG phase I	27 (10.5)	34 (15.0)
ELISA IgG phase II	29 (11.3)	35 (15.4)
ELISA IgM phase II	34 (13.2)	15 (6.6)
IFN-γ assay	161 (66.5)	122 (59.5)

Results of all serological assays were available of 257 subjects at six months and of 227 subjects at twelve months after vaccination. Results of IFN-γ assay were available of 255 subjects at six months and 227 subjects at twelve months after vaccination, but only subjects with conclusive results were considered (242 and 205 subjects at six and twelve months, respectively).
Abbreviations: IFA, immunofluorescence assay; CFT, complement fixation test; ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon-gamma.
[a] Positive is defined as IFA titre of ≥ 1:32, CFT titre of ≥ 1:10, ELISA IgG phase II titre ≥ 30 U/l, ELISA IgG phase I and IgM phase II ≥ extinction, *C. burnetii* specific IFN-γ production > 32 pg/mL.
[b] Either IgG or IgM, either phase I or phase II.

Fig. 3a shows the results of the IFN-γ assay after vaccination stratified according to the results of the IFA in patients in whom both tests were performed. Overall, the IFN-γ assay showed poor agreement with IFA: Fifty-nine percent ([85+56]/240, κ=0.197) after six months and 59% ([80+40]/202, κ=0.155) after twelve months. Only 35% of the vaccinees had both a detectable antibody and a cell-mediated immune response six months after vaccination and 40% twelve months after vaccination (Fig. 3b). Twenty-three percent (56/240) of the vaccinees had neither antibody nor IFN-γ immune response six months after vaccination, and twelve months after vaccination this was still 20%.

After logistic regression analysis, we found that at six months the absence of a detectable IFN-γ response after vaccination was related to use of immunosuppressive

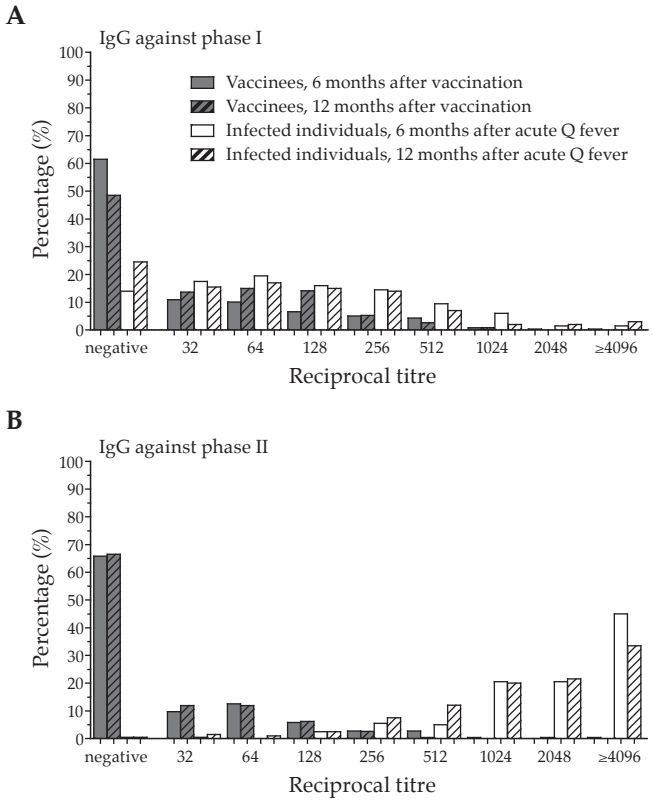

Figure 2. IgG titres against *C. burnetii* phase I and II antigens in vaccinated subjects as compared to individuals with past natural acute Q fever. IgG titres as measured by immunofluorescence (IFA). Percentage of individuals with respective IgG titre are shown for vaccinated (gray) and naturally infected individuals (white), six months (not-shaded) and twelve months (shaded) after vaccination or natural infection. (A) IgG antibodies against *C. burnetii* phase I, (B) IgG antibodies against *C. burnetii* phase II.

drugs: the IFN-γ response was absent in 73% (8/11) of the individuals using immunosuppressive drugs as compared to 36% (86/242) in individuals not on these drugs ($p = 0.02$). The serological response as measured by IFA was also more frequently absent: 82% (9/11) in vaccinees using immunosuppressive drugs compared to 53% (129/242) in individuals not on these drugs, although this did not reach statistical significance ($p = 0.08$). Higher age (>75 years) was also associated with an absent IFN-γ response: 61% (20/33) in subjects over 75 years of age compared to 34% (74/220) in subjects 75 years or younger ($p < 0.01$). This was not observed for the antibody response: 61% (20/33) absent in the older subjects vs. 54% (118/220) in the

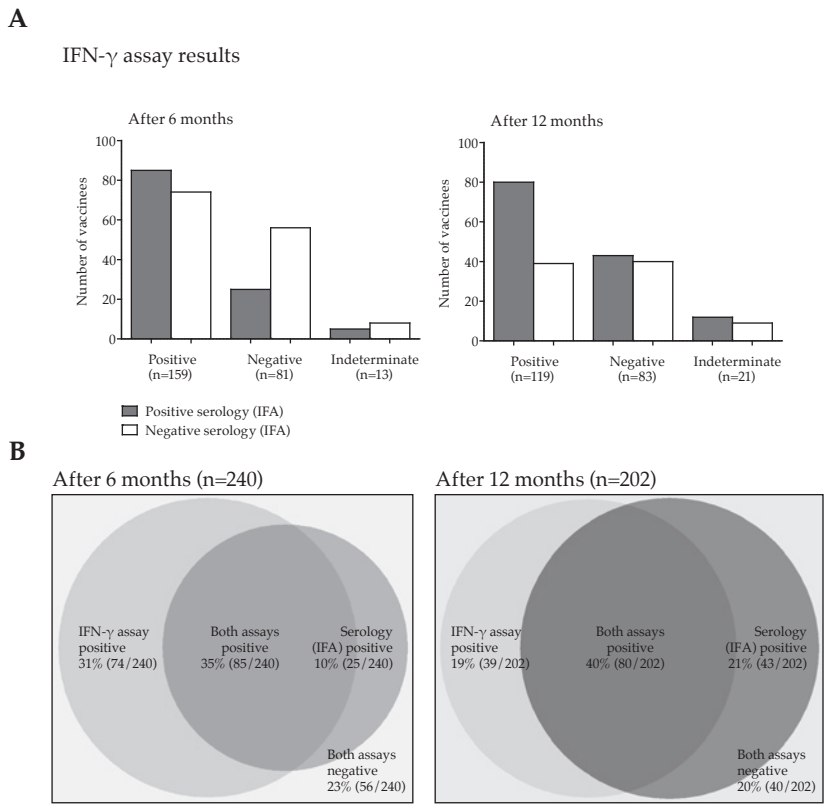

Figure 3. Results of IFN-γ assay vs. results of IFA in vaccinees 6 and 12 months after vaccination. (A) Interferon-gamma (IFN-γ) assay was considered positive when net *Coxiella burnetii*-specific IFN-g production was ≥32 pg/mL. IFN-γ assay was considered inconclusive when IFN-γ production in the positive aliquot did not exceed 24 pg/mL in combination with absent *C. burnetii*-specific IFN-γ production. Immunofluorescence assay (IFA) was considered positive when anti-*C. burnetii* antibody titre (either IgG or IgM, either against phase I or II organism) was ≥1:32. Results of both the IFN-γ assay and IFA were available of 253 individuals at 6 months after vaccination and of 223 individuals at 12 months after vaccination. (B) Venn-diagram showing overlap between positive results of IFN-γ assay and IFA at six and twelve months post-vaccination. Indeterminate results of IFN-γ assay are left unconsidered.

younger subjects (p = 0.45). Neither diabetes mellitus nor malignancy had a significant effect on cellular or humoral immune response to Q-vax vaccination.

Excluding the individuals taking immunosuppressive drugs, 22% percent (50/231) of the vaccinees had neither antibody nor IFN-γ immune response six months after vaccination, and twelve months after vaccination this was 18%.

Table 3 Comparison of immune response to vaccination between vaccinees with negative IFN-γ assay results prior to vaccination and vaccinees with positive IFN-γ assay results prior to vaccination.

	Vaccinees with negative IFN-γ assay prior to vaccination (n=197)	Vaccinees with positive IFN-γ assay prior to vaccination (n=42)	*P* value [d]
Pre-vaccination			
Positive serology (IFA)	0/197 (0%)	0/42 (0%)	NA
Positive IFN-γ assay	0/197 (0%)	42/42 (100%)	NA
PHA-induced IFN-g production (pg/mL)[a]	488 ± 74	425 ± 98	0.71[e]
C.b. NM induced IFN-γ production (pg/mL)[a]	4.2 ± 0.5	116 ± 18	NA
6 months after vaccination			
Positive serology (IFA)	80/194 (41%)	31/42 (74%)	<0.001
Positive IFN-γ assay[b]	114/186 (61%)	34/39 (87%)	0.002
C.b. NM induced IFN-γ production (pg/mL)[a,b]	105 ± 13	371 ± 115	<0.001[e]
12 months after vaccination			
Positive serology (IFA)	99/175 (57%)	30/36 (83%)	0.003
Positive IFN-γ assay[c]	87/155 (56%)	30/36 (83%)	0.002
C.b. NM induced IFN-γ production (pg/mL) [a,c]	67 ± 7	288 ± 81	<0.001[e]

Abbreviations: IFN-γ, interferon-gamma; IFA, immunofluorescence assay; NA, not applicable; *C.b.* NM, *Coxiella burnetii* Nine Mile.

[a] Mean ± SE net IFN-γ production after in vitro stimulation with PHA or *C. burnetii* Nine Mile.

[b] Only vaccinees that were tested with the IFN-γ assay six months after vaccination (n=194 and n=40) and with conclusive IFN-γ assay results (n=186 and n=39) are included.

[c] Only vaccinees that were tested with the IFN-γ assay twelve months after vaccination (n=173 and n=38) and with conclusive IFN-γ assay results (n=155 and n=36) are included.

[d] Fisher's exact test, unless otherwise indicated.

[e] Independent-samples *t* test.

Response to vaccination in subjects with or without pre-vaccination IFN-γ production

The 197 vaccinees with a negative IFN-γ assay pre-vaccination, did not significantly differ in terms of gender, age and comorbidity from the 42 vaccinees with a positive IFN-γ assay pre-vaccination (Table 1). However, as is shown in Table 3, the antibody and IFN-γ response after vaccination did differ between these two groups. A smaller proportion of vaccinees with a negative IFN-γ test pre-vaccination was seropositive at six and twelve months (41% and 57%) as compared to those with IFN-γ production pre-vaccination (74% and 83%; $p < 0.001$). The geometric mean reciprocal titre at six months was also significantly lower in the vaccinees with a negative IFN-γ test than in those with IFN-γ production pre-vaccination (28.8 vs. 62.9 and 24.8 vs. 52.2 for IgG against phase I and II, respectively; $p < 0.001$). Moreover, the mean quantitative increase in *C. burnetii*-specific IFN-γ production from pre-vaccination baseline was significantly lower in the vaccinees without pre-vaccination IFN-γ production (97 ± 12 pg/mL at six months and 56 ± 6 pg/mL at twelve months) than in those with pre-vaccination IFN-γ production to *C. burnetii* (245 ± 114 pg/mL and 166 ± 79 pg/mL) ($p < 0.05$).

Discussion

In the present study, we showed that the humoral and cellular immune responses after Q fever vaccination (Q-vax) in older adults with risk factors for chronic Q fever is relatively modest: 46 and 60% had antibodies against *C. burnetii*, at six and twelve months after vaccination, respectively, while 67 and 60% had a positive IFN-γ assay at these time points. Overall, 23% of the vaccinees (57/242) did not show any immune response at six months, which was associated with the use of immunosuppressive drugs and advanced age. The serological titres achieved by vaccination are much lower than seen after a natural infection. Likewise, the mean IFN-γ production in vaccinees at six months (141 ± 21 pg/mL) was considerably lower than previously found in individuals with earlier natural Q fever infection (449 ± 82 pg/mL).[21]

We evaluated three different serological techniques to measure the humoral immune response. IFA appeared to be the most sensitive method to detect specific antibodies after vaccination. This is in accordance with previous findings, suggesting that IFA should be used as the reference serological method for detecting natural infection.[23] Also in line with other studies,[16,24] is that vaccination is accompanied by emergence of antibodies against both phase I and phase II antigens. Although the vaccine is said to contain bacteria expressing phase I antigens, this is not surprising. Phase I cells also contain phase II antigens, as the

latter are an integral part of the normal phase I bacteria. Therefore, antibodies against both phases could be expected, comparable to the response after a natural infection (with phase I bacteria). The IgG response against phase II antigens seems more consistent over time than the IgG response against phase I antigens. The concordance between 6 and 12 months was 86% for IgG anti-phase II, and 77% for IgG anti-phase I (data not shown).

We found a lower humoral and cellular response to Q-vax than previously published. The first studies from Australia in abattoir employees, farmers and veterinarians reported seroconversion in 65% of the individuals 10e120 days after vaccination.[24] Later studies, reviewing the experience from 1981 to 1988 with Q-vax in more than 4000 vaccinees, reported a seroconversion rate of 84% up to 3 months after vaccination.[16] Similarly, Izzo et al. found in a much smaller sized study, a seroconversion rate of 80% after 2-4 weeks.[15] Whereas in this latter study the number of seropositive individuals declined with time after vaccination, the cell-mediated immune response (as measured by a lymphoproliferation assay) remained positive in 95% of vaccinees.[15,16] A recent small sized study from the USA reported a positive IFN-γ production assay in 81% (13/16) and a positive serology in a similar proportion.[25]

There are a number of explanations for the observed lower immune response in the present study. The higher long-lasting humoral response in Australian abattoir workers might be due to boosting by regular exposure to *C. burnetii* at the work site. Our study was performed after the acute Q fever outbreak had subsided, implicating that continuous exposure in the community was absent.[19] Probably of more importance, however, are the differences in the vaccinated populations. The Australian vaccinees were young and healthy abattoir workers, whereas our cohort was composed of elderly individuals with cardiovascular disease and other types of comorbidity. In fact, the use of immunosuppressive drugs (4.2%) and an age over 75 years old (13.1%) were associated with a diminished cellular vaccine response, and in case of immunosuppressive drugs also with the humoral response. A blunted IFN-γ immune and humoral response at higher age has been reported for other vaccines, e.g., influenza vaccine, and appears to be related to the degree of disability and comorbidity.[26,27] In general, the lower vaccine response in the elderly is considered a sign of immunosenescence, i.e., the decline in T- and B-cell function at older age.[28,29] On the other hand, individuals after a natural *C. burnetii* infection in the control group over 75 years of age ($n = 13$), had geometric mean reciprocal IgG titres against phase I of 2160 and phase II of 1655, which were not different from the younger individuals ($n = 187$). This suggests that exposure to presumably large numbers of live *C. burnetii*, possibly resulting in the persistence of non-infective *C. burnetii* antigens,[30] induces a more pronounced immune response than vaccination, even in the elderly. Therefore, it is tempting to speculate

that a higher dose of antigen in the vaccine might be needed to induce a more robust immune response in older people.

Chronic Q fever is characterized by very high titres of antibodies against *C. burnetii*, especially towards phase I antigens. Therefore, it can be argued that antibodies are not necessarily important for protection.[31] Nevertheless, it has been shown in mice that antibodies contribute in some way to protection against *C. burnetii* infection.[32-34] Cell-mediated immune responses are believed to be pivotal in the defense against intracellular pathogens including *C. burnetii*. [33,35,36] Thus, measuring cellular immune responses may be relevant to understand immune mechanisms in *C. burnetii* infection. However, our previous finding of high IFN-γ production in chronic Q fever [Schoffelen et al., manuscript submitted] does not point to a protective effect either. Thus, a direct relation between the humoral and cellular immune response after vaccination and the degree of protection will be difficult to establish.

Our study population was at risk for severe morbidity and mortality by chronic Q fever. This made it highly relevant to offer them protection against infection by vaccination. Data from Australia showed a high vaccine efficacy. In 924 vaccinated abattoir workers there was no evidence of Q fever at 18 months post-vaccination[11] and in a review of 4000 vaccinated individuals, there was strong suggestion that the eight cases of Q fever after vaccination all occurred in individuals vaccinated during the incubation period of natural *C. burnetii* infection. [16] Furthermore, a five-year follow-up study demonstrated that only two patients from a cohort of 2553 vaccinees developed breakthrough Q fever.[18] Finally, a recent meta-analysis by Gefenaite et al., showed an effectiveness of Q-vax of at least 97%.[17] Unfortunately, there is no data that demonstrate a relationship between the protection and the degree of immune responses, either humoral or cellular, after Q-vax vaccination. Because the Q fever epidemic in the Netherlands subsided just the year before the vaccination campaign, the present study will also be unable to deliver such data.

In our study, all subjects that were vaccinated had been seronegative as well as skin test negative beforehand.[19] Interestingly, the group with *C. burnetii*-specific IFN-γ production before vaccination showed a significantly higher seroconversion rate (74%) as compared to the negative IFN-γ production group (41%). The easiest explanation for this phenomenon is that there is a boosting effect of the vaccine in these subjects, reflecting previous infection not detected by serology or skin test. Alternatively, one could assume that these are high responders and it is non-specific IFN-γ production not necessarily a result of previous exposure to *C. burnetii*. However, the control PHA-induced IFN-γ in these individuals did not differ from those without *C. burnetii*-specific IFN-γ response. Assuming that specific IFN-γ production is caused by a previous infection, this implies that a positive IFN-γ

production could be of additional value in pre-vaccination screening, excluding potential vaccinees with pre-existing immunity. It is likely that the IFN-γ test identified subjects who had been exposed to *C. burnetii*, but failed to mount a measurable antibody response and skin reaction. This extends the findings of our pre-vaccination screening study, in which we compared the IFN-γ test with both serology and skin test, and concluded that the IFN-γ test had similar sensitivity and specificity as the combination of both other tests and has additional value when the other tests have borderline results.[21] To what extent a positive IFN-γ response in pre-vaccination screening predicts adverse reaction to vaccination, is currently under investigation.

In conclusion, the antibody and IFN-γ responses after Q fever vaccination in an elderly population at risk for chronic Q fever is lower and restricted to a smaller proportion of vaccinees than found in a control group with past Q fever and than previously described in other vaccinated cohorts. Studies in similar risk groups, that remain exposed to *C. burnetii* after vaccination, are needed to gain insight in the relation between humoral and cellular immune responses and vaccine efficacy i.e., protection.

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Immunogenicity of the Q fever skin test

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Abstract

Objectives: The Q fever skin test is used to measure cell-mediated immunity to *Coxiella burnetii* in pre-vaccination screening to exclude individuals with pre-existing immunity. We investigated whether this in-vivo test influences subsequent measurements of immune response.

Methods: We assessed the humoral and cellular immune responses before, and 6 and 12 months after skin testing in 63 individuals who were not vaccinated because of either a positive skin test or positive serology in screening. IgG anti-*C. burnetii* antibodies were measured using immune-fluorescence assay (IFA). The cellular immune response was assessed by measuring in-vitro *C. burnetii*-specific interferon (IFN)-γ production in blood.

Results: Of the 35 subjects with a positive skin test and negative serology, 15/35 (43%) showed seroconversion at 6 months, and 7/32 (22%) seropositivity at 12 months. The mean ± SE specific IFN-γ production in this group increased from 185 ± 88 pg/mL (at baseline) to 422 ± 141 pg/mL at 6 months ($P = 0.009$) and 223 ± 91 pg/mL at 12 months ($P = 0.17$). Of the 28 subjects with positive serology (and unknown skin test results), 21/28 (75%) showed an increase in IgG anti-phase I titres at 6 months, and 11/25 (44%) at 12 months. The mean ± SE specific IFN-γ production was significantly increased at 6 months, but not at 12 months.

Conclusions: Q fever skin testing causes higher antibody titres and higher in-vitro IFN-γ to *C. burnetii*, and therefore affects subsequent Q fever diagnostics.

Introduction

Q fever is caused by *Coxiella burnetii*, an intracellular Gram-negative bacterium. Initial infection often goes unnoticed, but in a minority of cases chronic infection develops and can present as endocarditis or vascular wall infection, which leads to high morbidity and mortality [1]. Infection is normally diagnosed by serology, by measuring IgM and IgG antibodies against the two different antigenic phases of *C. burnetii*.

Q fever skin testing is used to measure in vivo cell-mediated immunity to *C. burnetii*. Its use is limited to pre-vaccination screening, to exclude individuals with pre-existing immunity who would be at increased risk of hypersensitivity reactions to the vaccine [2]. Major drawbacks of the skin test are that it requires specialized personnel, needs a second visit to read, and can be troublesome for the tested subjects. Even more important, from an immunological point of view and with consequences for (epidemiological) follow up, is that in-vivo skin testing, by injection of *C. burnetii* antigens, may induce a cellular and humoral immune response and thus possibly modifies subsequent immunologic measurements.

Between 2007 and 2010, the Netherlands faced the largest Q fever outbreak ever reported, with more than 4000 notified human cases of acute Q fever [3]. In June 2010, the Dutch health authorities decided to offer vaccination to all individuals at risk for chronic Q fever living in the area of the epidemic. The resulting screening and vaccination took place between January 2011 and July 2011 [4].

In the current study, we evaluated the impact of skin testing on the measurement of both humoral and cellular *C. burnetii* specific immune responses in a group of 63 individuals who were screened in a Q fever vaccination campaign and were not subsequently vaccinated.

Subjects and methods

Setting

The study was performed during a Q fever vaccination campaign in the Netherlands (January-July 2011) and the year thereafter. Before Q fever vaccination, all 1786 eligible individuals were screened by skin testing and Q fever serology: 210 were found to be skin test positive (but seronegative) and 181 were seropositive (with unknown skin test results, because they were not invited for reading). In accordance with the recommendations, both groups were excluded from vaccination because of the increased risk of side effects [2]. Individuals from these excluded groups were asked to participate in the post-vaccination study. The study was approved by the Central Committee on Research on Human Subjects (CCMO).

After informed consent, blood was collected just before, and 6 and 12 months after skin testing to measure the humoral and cellular specific immune responses to *C. burnetii*.

Q fever skin test

The commercially available Q-vax® skin test (CSL, Australia) was used [5]. It contains diluted formaldehyde-inactivated *C. burnetii* Henzerling strain phase I-antigens (10 times lower concentration than in Q-vax® vaccine). One dose of Q-vax skin test (lot# 0996-06501) was diluted 30 times and 0.1 mL was administered intracutaneously in the forearm according to the product information [6]. The local reaction to the skin test was read after seven days, but only if serology was negative. Any localized induration was considered to be positive and led to exclusion from vaccination.

Q fever serology

The humoral immune response was assessed by a commercially available immunofluorescence assay (IFA; Focus Diagnostics, Cypress, USA). A positive serology was defined as anti-phase I or phase II *C. burnetii* IgM/IgG titre ≥1:32. An increase of titres was defined by two-fold increase or more.

C. burnetii specific IFN-γ production

Cellular immune response was assessed by measuring in-vitro *C. burnetii* specific IFN-γ production in whole blood as previously described.[7] Net IFN-γ production was expressed as the concentration of IFN-γ in the *C. burnetii* stimulated sample minus that in the negative control sample. The assay was considered positive when net IFN-γ production was ≥32 pg/mL, based on previous findings [7].

Statistics

Mean ± standard error (SE) IFN-γ production was calculated and compared using a paired samples *t*-test. Median serological titres were calculated for groups and the Wilcoxon matched-pairs signed rank test was used to compare median serological titres at different time points. $P < 0.05$ was considered significant.

Results

Sixty-three individuals (mean age [±SD] of 66 [±11] years, 76% male) were asked to participate in the study: 35/63 with a positive skin test and 28/63 with positive serology and unknown skin test results, at screening. These were selected from the larger group of eligible individuals, because they lived close to the health care

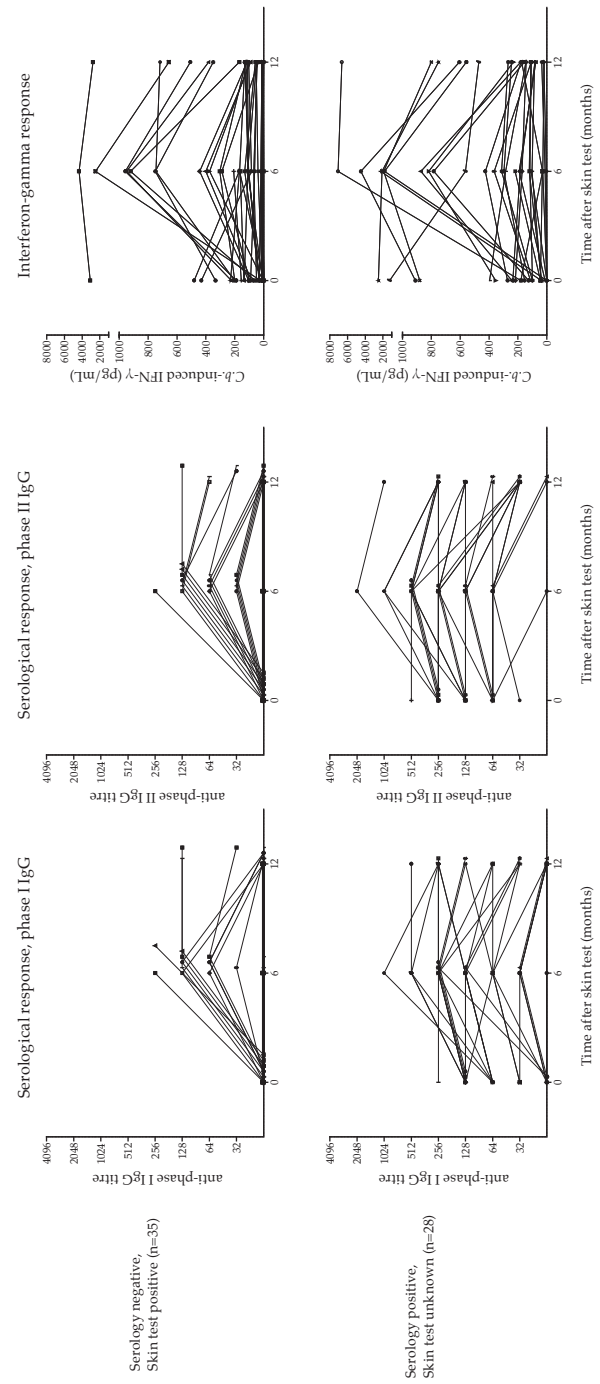

Figure 1. Individual immune responses, both serological and interferon-gamma (IFN-γ), of all study subjects before (t = 0) and at 6 and 12 months after Q fever skin test. The subjects with negative serology but positive skin test (n = 35) are depicted separately from those with positive serology and unknown skin test (n = 28) in pre-vaccination screening. Serological response is shown as the anti-*C. burnetii* phase I and II IgG titre, as measured with immunofluorescence assay. *C.b.*-induced IFN-γ is the net in-vitro IFN-γ production in pg/mL after stimulation of blood with *C. burnetii* Nine Mile minus IFN-γ in the unstimulated sample.

centre where blood was collected. At twelve months, 32/35 and 25/28 of these individuals were available for repeated blood tests.

Individual immune responses at baseline, 6 and 12 months are shown in Fig. 1. Of the 35 individuals with a positive skin test and negative serology, 15/35 (43%) showed seroconversion after 6 months, and 7/32 (22%) were seropositive after 12 months. The proportions with positive results in the IFN-γ assay were 19/35 (54%) at baseline, 26/35 (74%) after 6 months and 22/32 (69%) after 12 months. The mean ± SE specific IFN-γ production in this group increased from 185 ± 88 pg/mL (at baseline) to 422 ± 141 pg/mL at 6 months ($P = 0.009$) and 223 ± 91 pg/mL at 12 months ($P = 0.17$) after skin testing. In the 28 individuals with positive serology in pre-vaccination screening, an increase of IgG anti-phase I and II antibody titre was observed after the skin test: at 6 months, 21/28 (75%) had an increase in phase I IgG titre, 14/28 (50%) in phase II IgG titre; at 12 months, 11/25 (44%) in phase I IgG and 4/25 (16%) phase II IgG titre. The increase of median titre was 2-fold after 6 months (median phase I IgG from 1:64 to 1:128, $P = 0.0001$; phase II IgG from 1:128 to 1:256, $P = 0.001$) and this significant increase largely disappeared after 12 months (median phase I IgG 1:64 $P = 0.04$; phase II IgG 1:64 $P = 0.20$). The proportions with positive results in the IFN-γ assay were 22/28 (79%) at baseline, 23/28 (82%) after 6 months and 22/25 (88%) after 12 months. The IFN-γ production in this group increased from 301 ± 100 pg/mL (at baseline) to 918 ± 296 pg/mL at 6 months ($P = 0.039$) and 500 ± 271 pg/mL 12 months ($P = 0.598$) after skin testing.

Discussion

Our results showed that the intracutaneous Q fever test, containing a low dose of Q-vax antigen, induces clear *C. burnetii*-specific immune responses in about half of the tested individuals with a positive Q fever skin test. In addition, a median 2-fold increase in antibody titre was found in patients with positive serology in pre-vaccination screening. These effects were measurable after 6 months, but seemed to wane at 12 months. Since all individuals in this study had evidence of pre-existing immunity (either a positive skin test or positive serology), this immune response should be considered as a boosting effect.

Strikingly, the proportion of individuals who seroconverted at 6 months after a positive skin test (43%), was almost as high as what was previously reported at 6 months after Q fever vaccination with a 300 times higher dose of individuals with a negative skin test (46%) [8]. In addition, the previously reported mean ± SE IFN-γ production in vaccinated individuals was 22 ± 4 pg/mL (at baseline), 141 ± 21 pg/mL at six months and 94 ± 15 pg/mL at 12 months after Q fever vaccination. Thus, while the IFN-γ production was already higher in the skin-test positive

subjects than in the vaccinated individuals, vaccination reached similar levels as in the skin-test positive subject at baseline, while the latter reached a mean IFN-γ production of more than twice as high. In individuals who were seropositive in pre-vaccination screening, skin testing also led to a boosting effect, increasing the anti-*C. burnetii* IgG titres as measured after 6, but not after 12 months.

A similar boosting effect is described for the tuberculin skin test (TST) on subsequent interferon-gamma release assay (IGRA) responses, often used in combination to diagnose latent tuberculosis[9]. This phenomenon makes it difficult for clinicians to discriminate between boosting and conversion of any increase in the IGRA response after TST.

The immunogenetic effect of the Q fever skin test has been described before, but has never been quantified. Intracutaneous administration of undiluted Q fever whole-cell vaccine has been tried in imprisoned volunteers in the early 1960s [10]. The antibody response appeared to be related to the number of doses of vaccine given, but was more delayed and reached lower titres than after subcutaneous administration. Moreover, five out of six skin test positive individuals, who subsequently were not vaccinated, seroconverted in the follow-up. However, the problem of repeated injection of whole-cell vaccines, is the increased risk for developing adverse reactions. To overcome this problem, Waag et al. used a chemical extraction procedure to develop a vaccine with good immunogenicity and reduced adverse effects upon serial use. They showed in non-immune volunteers that a second subcutaneous vaccination with a chloroform-methanol residue phase I vaccine, 3-6 months after a priming subcutaneous dose, can mount serological and cellular response, whereas one dose did not [11].

In this respect, the intracutaneous Q fever test can be seen as a kind of vaccination. In rabies prophylaxis, intradermal administration of cell culture vaccines is an accepted alternative to the standard intramuscular route [12]. It only takes one-tenth of the dose that is used intramuscularly to obtain an adequate humoral response [13]. The effective skin-associated immune response is most likely due to the extensive local network of dendritic cells and the skin-draining lymph nodes to generate effector T and B cells, leading to long-term protective immunity.

We believe that clinicians, immunologists and epidemiologists should be aware of the phenomenon that individuals who have been skin tested, as is the current practice in Q fever pre-vaccination screening, may develop higher anti-phase I *C. burnetii* IgG antibody titres and a more robust in-vitro IFN-γ release. This may lead to false diagnosis of re-infection or even possible chronic Q fever. We also suggest that an in-vitro test, like the *C. burnetii* specific IFN-γ production assay, should be further investigated as an alternative for the Q fever skin test for assessing pre-existing immunity.

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Coverage of the 2011 Q fever vaccination campaign in the Netherlands, using retrospective population-based prevalence estimation of cardiovascular risk-conditions for chronic Q fever

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Abstract

Background: In 2011, a unique Q fever vaccination campaign targeted people at risk for chronic Q fever in the southeast of the Netherlands. General practitioners referred patients with defined cardiovascular risk-conditions (age ≥15 years). Prevalence rates of those risk-conditions were lacking, standing in the way of adequate planning and coverage estimation. We aimed to obtain prevalence rates retrospectively in order to estimate coverage of the Q fever vaccination campaign.
Methods: With broad search terms for these predefined risk-conditions, we extracted patient-records from a large longitudinal general-practice research-database in the Netherlands (IPCI-database). After validation of these records, obtained prevalence rates (stratified for age and sex) extrapolated to the Q fever high-incidence area population, gave an approximation of the size of the targeted patient-group. Coverage calculation addressed people actually screened by a pre-vaccination Q fever skin test and serology (coverage) and patients referred by their general practitioners (adjusted-coverage) in the 2011 campaign.
Results: Our prevalence estimate of any risk-condition was 3.1% (lower-upper limits 2.9-3.3%). For heart valve defects, aorta aneurysm/prosthesis, congenital anomalies and endocarditis, prevalence was 2.4%, 0.6%, 0.4% and 0.1%, respectively. Estimated number of eligible people in the Q fever high-incidence area was 11,724 (10,965-12,532). With 1330 people screened for vaccination, coverage of the vaccination campaign was 11%. For referred people, the adjusted coverage was 18%. Coverage was lowest among the very-old and highest for people aged 50-70 years.
Conclusion: The estimated coverage of the vaccination campaign was limited. This should be interpreted in the light of the complexity of this target-group with much co-morbidity, and of the vaccine that required invasive pre-vaccination screening. Calculation of prevalence rates of risk-conditions based on the IPCI-database was feasible. This procedure proved an efficient tool for future use, when prevalence estimates for policy, implementation or surveillance of subgroup-vaccination or other health-care interventions are needed.

Introduction

In 2007, a large human Q fever outbreak started in the southeast of the Netherlands, caused by exposure to goat-farms infected by *Coxiella burnetii* [1]. Subsequently, the annual number of reported human Q fever cases reached a peak of 2354 in 2009. The increase of acute Q fever reports was followed by increasing numbers of chronic Q fever patients [2]. Chronic Q fever is a severe outcome of *C. burnetii* infection, often presenting itself as endocarditis or vascular infection, and needs long-term antibiotic treatment and often surgical intervention [3]. Several cardiovascular risk-conditions for chronic Q fever have been identified [4,5], and the Health Council (HC) of the Netherlands advised vaccination of people aged 15 years and over, with these specific risk-conditions in July 2010 [6]. This measure was an individual patient-oriented intervention and not a population-targeted campaign. The sole available vaccine, only registered in Australia where it is used to vaccinate people at occupational risk [7,8], requires pre-vaccination screening [9,10], and therefore complicated logistics [11]. The Ministry of Health chose a centralized approach, coordinated by the National Institute for Public Health and Environment. Efforts to reach the target population concentrated on high-incidence areas [Fig. 1] and relied on general practitioners (GPs), and to a lesser extent to hospital specialists, for patient referrals. Intake, screening and subsequent vaccination was organized at the municipal health clinic in the central high-incidence area. The vaccination campaign included intensified safety surveillance [12], immunological pre-vaccination screening and immunological follow-up [11,13].

In their advice, the HC did not attempt to quantify the size of the risk-group eligible for vaccination and no concise prevalence data of the risk-conditions were available according to cardiologists. In the planning phase of the vaccination campaign, which should start before the approaching lambing season, it was estimated that 100-300 people would respond to the invitation for vaccination within the target population, and 1000 was taken as upper limit budget wise. The much larger than expected number of referred people (2741) overloaded the system and made implementation of the campaign difficult, forcing an up-scaling of staff [11]. In the end, 1786 eligible people underwent screening tests between January and April 2011.

The lack of prevalence data of the risk-conditions not only precluded determination of the size of the target population, but also of the coverage of the vaccination campaign. In order to estimate coverage retrospectively, we needed to obtain prevalence estimates of the risk-conditions for chronic Q fever in the high-incidence area. To this end, we extracted data on the specific risk-conditions from the Integrated Primary Care Information (IPCI)-database, a large longitudinal population-based general practice research database in the Netherlands. Here we

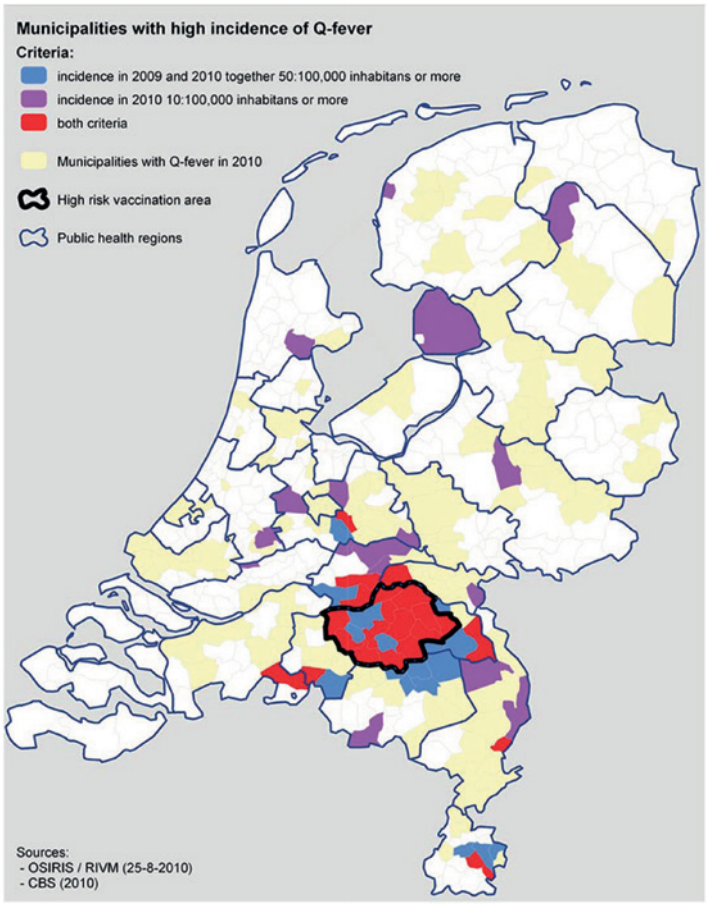

Figure 1. Municipalities with their incidence of Q fever in 2009-2010. Q fever high-incidence area outlined in black (11).

describe the procedure we followed to arrive at an estimate of the coverage of the Q fever vaccination campaign in the Netherlands 2011 [14].

Materials and Methods

IPCI-database

The IPCI-database is a longitudinal general practice research database held by Erasmus University Medical Center, Rotterdam. With data collection since 1996,

this electronic dynamic database presently contains 1.1 million patient-records from 437 general practices in the Netherlands [15-17]. It holds comprehensive information on medical history and referrals. Included are signs and symptoms, diagnoses, International Classification of Primary Care (ICPC)-codes, laboratory test results, surgical procedures, medication and hospitalizations.

The free text fields of the records contain summaries or full discharge letters from hospital specialists. Hard copies of letters are available upon request. The patient population is representative of the Dutch population, with some under-representation of elderly people who have moved to nursing homes however [17]. Patient-records are anonymous for researchers. IPCI complies with European Union guidelines on secondary use of healthcare data for medical research. The IPCI- database is valid for pharmaco-epidemiological research and has been used in more than 100 peer-reviewed publications. The Supervisory Board of the IPCI-database (project number 11/2012) approved the use of IPCI-data for the current prevalence estimation. Approval by the ethics committee was not necessary for this retrospective study because only anonymous data have been used.

Study population

The source population comprised all patients 15 years and older who were registered with one of the GP-practices and actively in follow-up with the GP on January 1st 2011 [6]. Due to resource limitations regarding the necessary manual validation of extracted patient-records, we sampled 5% of this source population as study population. We compared the characteristics of the full source population and those of the 5% random sample for consistency in pattern of non-validated potential cases.

Risk-conditions and search criteria

Four risk-conditions were defined, based on the HC-advice of eligibility for Q fever vaccination [6]. Relevant search terms were entered in a script (syntax) with which the database was searched in diagnostic code sections and free text fields [Table 1]. Physicians (PEVdB and TS) reviewed the electronic medical records of all potential cases in order to assess validity and label the diagnostic certainty. They entered the level of diagnostic certainty (definite-probable-possible case or no case) in a validation tool with date of first diagnosis or of rejection. Cases could have multiple risk-conditions. We did not subdivide the cases according to severity or type of defect within the specific risk-conditions, since this was not the scope of our study. A random sample of 100 records was crosschecked by independent review (AMV). These records and all cases with the lowest level of diagnostic certainty (possible) were thereafter checked for consistency, held against strict inclusion criteria, and coded following consensus (PEVdB and TS).

Table 1 Cardiovascular risk-conditions for chronic Q fever with ICPC-codes, search terms and diagnostic certainty levels.

Risk-conditions, ICPC-codes[a], search terms and diagnostic certainty levels for IPCI-database[b] extraction		
Risk-conditions and ICPC-codes[c]	**1-**	valvular cardiac disease or prosthesis (symptomless mitral valve prolapse excluded) (K83)
	2-	aortic aneurysm of prosthesis/stent (K99)
	3-	congenital cardiac anomalies (except spontaneous closure of VSD/ASD/OBD or surgical closure without artificial material and no residual defect), inclusive of coarctatio aortae (K73)
	4-	history of endocarditis or rheumatic cardiac disease (K71)
Search terms in free text	**a-**	valv*, aort*, aneury*, congenit*, viti*, prosth*, vsd/asd, obd, mitra*, tricusp*, stenos*, insuff*, endocardi* and the Dutch equivalents, etcetera's
	b-	proper names of different (congenital) syndromes like Eisenmenger, Fallot, Epstein, Ivemark, Botalli, etcetera's. All these search terms included also possible spelling errors
Level of diagnostic certainty	**1-**	certain; with discharge letter of specialist or specific diagnostic laboratory outcome, or repetitive entries with description and specific treatment
	2-	probable; with only descriptions entered by the general practitioner, with the correct ICPC-codes
	3-	possible; with only (recurrent) ICPC-code, without description of condition, or minimal defects with uncertain consequences
	4-	rejected; if condition could be regarded as variant of normal, minimal defects without any consequences, or falling within exclusion criteria, as well as conditions excluded by specialist examination, or conditions in family members

[a] ICPC-codes, International Classification of Primary Care codes
[b] IPCI-database, Integrated Primary Care Information database
[c] Qualifying patients were eligible for Q fever vaccination in the campaign of 2011

Population of the Q fever high-incidence area and the Q fever vaccination campaign

The population distribution of the Q fever high-incidence area as of January 1st 2011 was retrieved from demographic data from the electronic databank Statline (Statistics Netherlands, http://statline.cbs.nl/Statweb). Information on actually referred and screened people in the Q fever vaccination campaign was obtained from the case report forms of the Q fever vaccination campaign as described

previously [12]. Risk-conditions of referred people came from GPs and from the potential vaccinees themselves. Postal codes identified referred people living in the high-incidence area. Of 2741 potential vaccinees, 955 were excluded for various reasons [11]. The remaining 1786 people –of which 1330 from the high-incidence area– underwent pre-vaccination screening with skin test and serology, and –if negative– were subsequently vaccinated. Logistics and safety surveillance of the Q fever vaccination campaign have been described elsewhere [11,12].

Data analysis

After validation and review of extracted patient-records from the IPCI-sample, stratified numbers on the four defined risk-conditions according to 10 years' age groups and sex, were used to determine prevalence (N/10,000), with 95% Confidence Intervals (95%CI). We did so both for all confirmed cases and for cases with definite and probable diagnostic certainty only, with lower and upper limits. The prevalence estimates for cases with definite and probable diagnostic certainty, stratified for age group and sex, served as input for the high-incidence area population, to approximate the number of people eligible for Q fever vaccination in this region. With this, the coverage of the vaccination campaign was determined with minimum and maximum estimates based on the 95% CI of the IPCI-sample data. Fig. 2 presents a diagram with the calculation steps. OpenEpi and Excel were used for calculations and graphs.

Results

The IPCI-database per January 1st 2011 contained 651,276 people aged 15 years and over. The 5% random sample as study population had comparable age and sex distribution. The relative age distribution of the populations of the IPCI-sample and of the high-incidence area (as obtained from CBS) shows that they were similar (with equal median age), though the latter had slightly less people in the age group of 20-30 years and more in the 50-60 year olds [Fig. 3].

The IPCI-study population included 32,571 people [Table 2]. We identified 1966 patient-records (6%) with one or more ICPC-codes and/or search terms for chronic Q fever risk-conditions [Table 1], women (52.7%) slightly outnumbering men (47.3%). After review and validation, we rejected 948 extracted patients in all (viz. hits for family members with a risk-condition, or medical investigation ruling out a specified risk-condition, or non-qualifying diagnoses). This left 1018 (52%) cases with one or more confirmed chronic Q fever risk-conditions. Of these, 177 (17%) had possible diagnostic certainty and 841 definite or probable diagnostic certainty [Fig. 4].

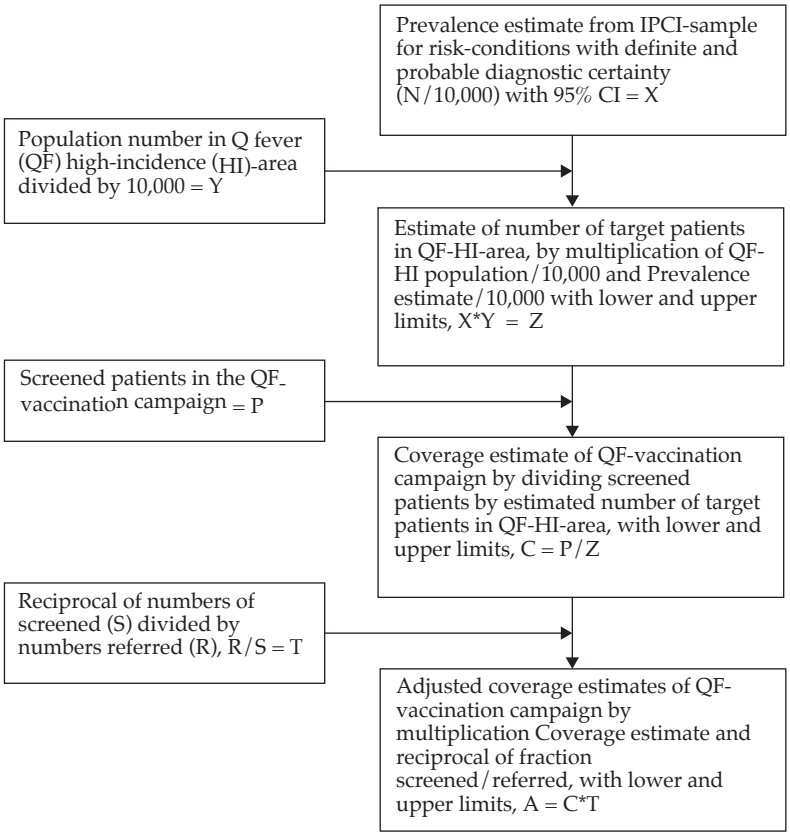

Figure 2. Flow diagram of calculations leading to coverage estimates. For calculations, prevalence of risk-conditions with definite and probable diagnostic certainty from IPCI-study population has been used, overall and for subgroups.

The majority of confirmed cases from the IPCI-study population (925/1,018; 91%) had only one defined risk-condition and 9.1% had multiple risk-conditions, mostly double and only three triple (male 11.5% and female 7.0%). Valvular disease was most prevalent (778; 148 possible), followed by aortic aneurysm or prosthesis (183; 10 possible), congenital cardiac anomalies (116; 28 possible) and endocarditis (37; 15 possible) [Table 2 and Fig. 4].

The relative frequencies of the four risk-conditions in the IPCI-study population were similar to those in the screened people of the Q fever campaign and the screened people from the high-incidence area [Fig. 5A]. However, the screened people were younger than the confirmed cases with definite and probable diagnostic certainty

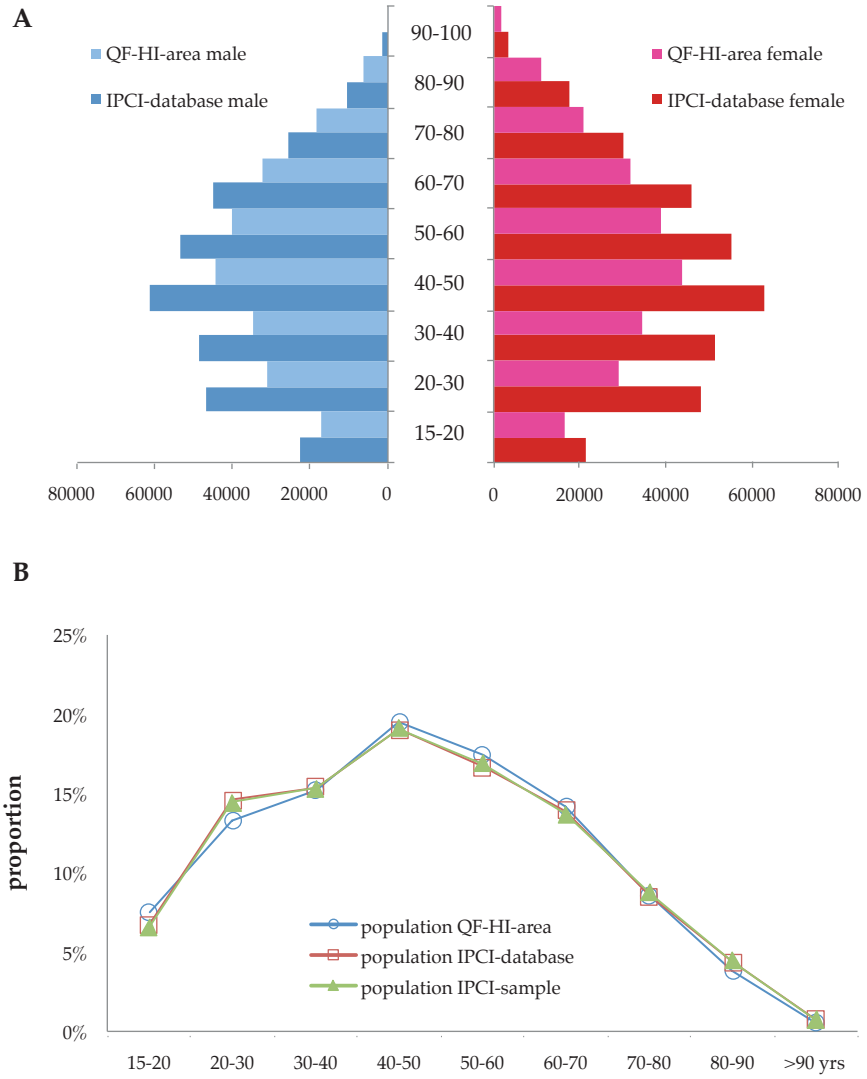

Figure 3. Population distribution of IPCI-database and of Q fever high-incidence area. (A) population pyramids for IPCI-database (dark colours) and for Q fever high-incidence (HI)- area (light colours), according to sex. (B) Relative age frequencies for IPCI-source population (open squares), IPCI-study population (triangles) and for Q fever HI-area (open circles).

Table 2 Risk-conditions for chronic Q fever in the IPCI-study population, according to age groups and sex. Distribution of the full IPCI-study population; number of confirmed risk-conditions in the IPCI-study population; prevalence estimates of risk-conditions per 10,000 according to sex, as calculated from the IPCI-study population, with lower and upper limits.

IPCI-population, risk-conditions, prevalence rates and age groups										
IPCI-study population (number)										
Age group	**15-20**	**20-30**	**30-40**	**40-50**	**50-60**	**60-70**	**70-80**	**80-90**	**>90 yrs**	**all**
all	2158	4708	5001	6224	5509	4454	2837	1447	233	32571
male	1093	2354	2400	3047	2728	2138	1300	579	72	15711
female	1065	2354	2601	3177	2781	2316	1537	868	161	16860
male%	51%	50%	48%	49%	50%	48%	46%	40%	31%	48%
Risk-conditions (number)[a]										
Age group	**15-20**	**20-30**	**30-40**	**40-50**	**50-60**	**60-70**	**70-80**	**80-90**	**>90 yrs**	**all**
Heart valve defect	3	9	14	54	67	166	225	199	41	778
Aortic aneurysm/prosthesis	1	2	0	7	19	38	63	49	4	183
Congenital heart anomaly	15	24	17	24	14	14	3	5	0	116
Endocarditis	0	1	3	4	7	12	6	4	0	37
Any risk condition	16	31	30	82	94	212	269	241	43	1018
male	6	16	11	34	44	111	142	110	13	487
female	10	15	19	48	50	101	127	131	30	531
male%	38%	52%	37%	41%	47%	52%	53%	46%	30%	48%

Prevalence of risk-conditions (/10,000)[a]										
Age group	**15-20**	**20-30**	**30-40**	**40-50**	**50-60**	**60-70**	**70-80**	**80-90**	**>90 yrs**	**all**
Heart valve defect	14	19	28	87	122	373	793	1375	1760	239
male	9	21	21	66	106	379	815	1347	1667	214
female	19	17	35	107	137	367	774	1394	1801	262
Aortic aneurysm/prosthesis	5	4	0	11	34	85	222	339	172	56
male	0	8	0	13	51	131	385	674	417	89
female	9	0	0	9	18	43	85	115	62	26
Congenital heart anomaly	70	51	34	39	25	31	11	35	0	36
male	55	51	29	39	22	33	15	17	0	34
female	85	51	38	38	29	30	7	46	0	37
Endocarditis	0	2	6	6	13	27	21	28	0	11
male	0	4	0	3	15	28	23	17	0	10
female	0	0	12	9	11	26	20	35	0	12
Any risk-condition	74	66	60	132	171	476	948	1666	1845	313
lower limit	46	46	42	106	140	417	846	1482	1400	294
upper limit	120	93	86	163	208	543	1062	1866	2393	332
male	55	68	46	112	161	519	1092	1900	1806	310
female	94	64	73	151	180	436	826	1509	1863	315

[a] The scoring of risk-conditions as shown here, applies to all confirmed cases, including possible and probable/definite diagnostic certainty.

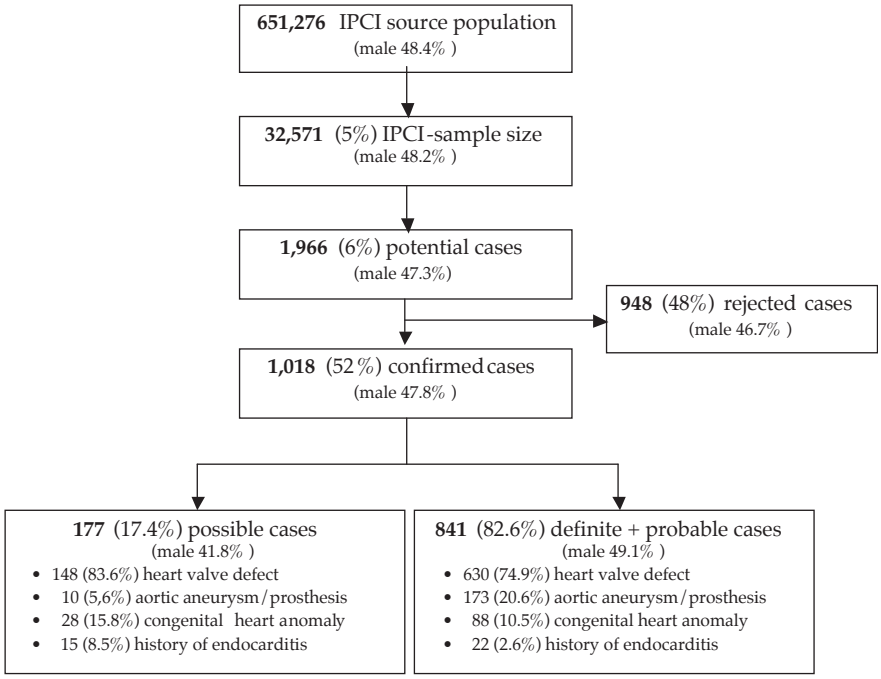

Figure 4. Flow diagram of results of IPCI-database extraction and validation. Sex distribution (male %) included for each step.

from the IPCI-study population. Median (interquartile range) ages were 67 (57-74) and 72 (61-81) years for screened people and IPCI-cases, respectively. IPCI-cases with possible diagnostic certainty were younger and had a higher proportion of women (58% versus 51%). The proportion of men was higher in the referred and screened people of the Q fever vaccination campaign from the high-incidence area (61%) than in the IPCI-study population cases (48%), for all risk-conditions except for congenital heart anomalies [Figs. 4 and 5B].

The overall prevalence of any risk-condition in the IPCI-study population was 313 per 10,000 people, increasing with age [Fig. 6]. Only for congenital anomalies, prevalence was highest in the youngest age group. Men and women showed almost equal overall prevalence rates of 310 and 315 per 10,000, respectively [Table 2]. These calculated prevalence rates for the different risk-conditions are within data ranges presented in literature [Table 3].

To get an impression of the proportion of eligible people who were reached in the Q fever vaccination campaign (coverage), one should first calculate prevalence

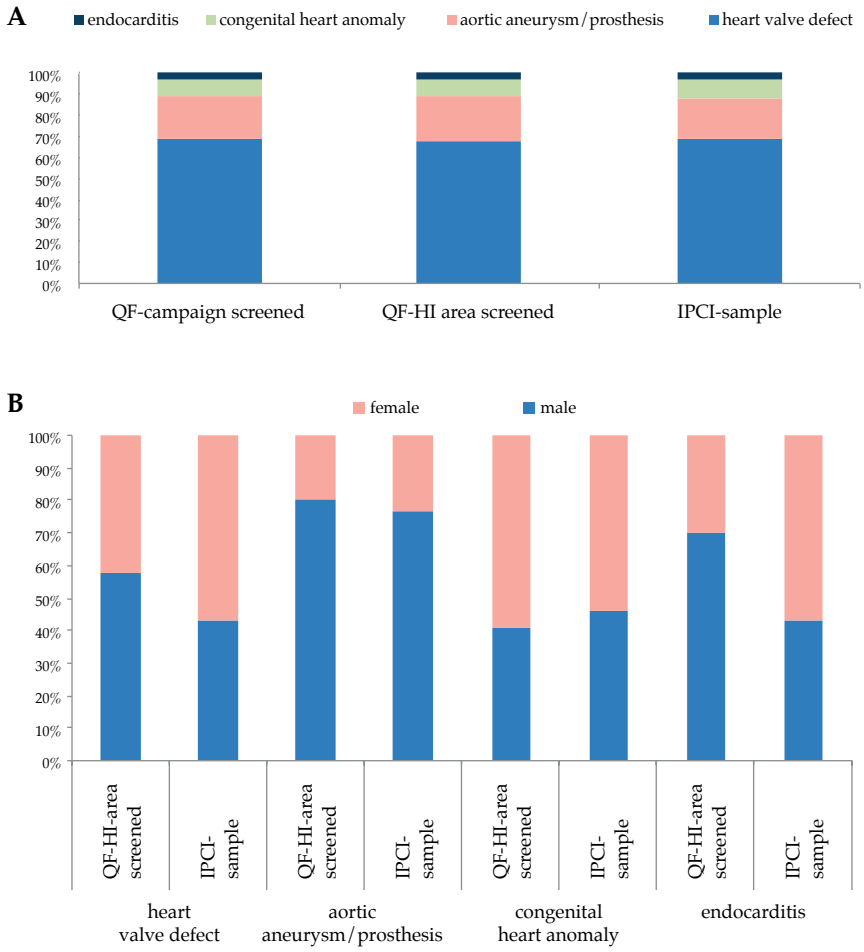

Figure 5. Relative frequencies of risk-conditions for chronic Q fever and sex distribution in the different populations. (A) Comparison between all screened people of the Q fever (QF)-vaccination campaign, screened people from the high-incidence (HI)-area, and cases from the IPCI-study population. (B) Sex distribution for different risk-conditions in screened people from QF-HI-area and cases from IPCI-study population. The IPCI-study population includes cases with definite and probable diagnostic certainty.

estimates for validated IPCI-cases with definite and probable diagnostic certainty [Fig. 2]. These prevalence rates were stratified for age group and sex, with an overall rate of 258/10,000 [S1 Table]. Extrapolation of these rates to the population of the Q fever-high-incidence area, led to an estimated number of targeted people of 11,724

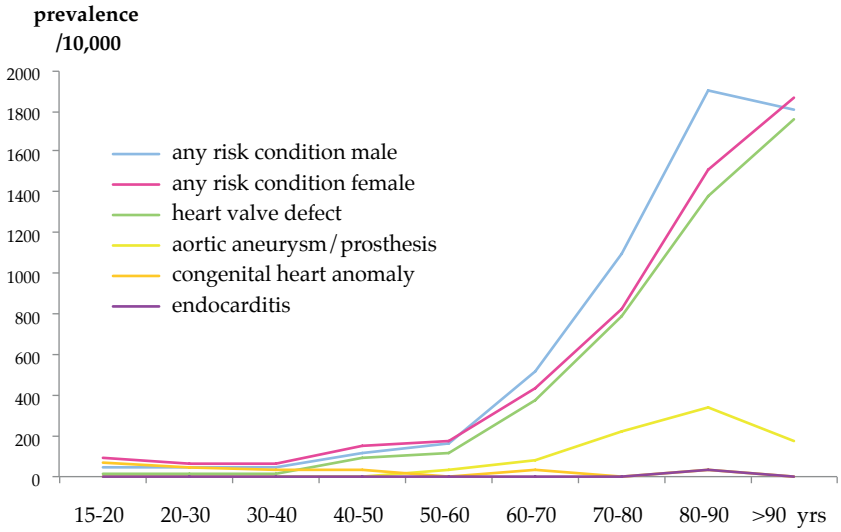

Figure 6. Age distribution of the defined risk conditions for chronic Q fever. Prevalence rates (per 10,000) are estimated from the IPCI-database, per January 1st 2011.

(10,965-12,532) [S2 Table]. If people from municipalities bordering the high-incidence area were included in this calculation, the target group would number at least 15,000 people.

Overall, only 1330 of this target group from the high incidence area were screened for vaccination [Table 4], which resulted in coverage of 11% (1330/11,724) with narrow lower and upper limits of 11-12%. For men and women these coverage rates were 14% (13%-15%) and 9% (8%-10%), respectively. For the larger group of referred people from the high-incidence area, an adjusted coverage rate of 18% (limits 16%-18%) was calculated. Coverage was lowest in the oldest age groups, with 5% and 1% for the 80-90 year olds and 90 years and older, respectively. The highest coverage was in the much larger groups of the 50-60 and 60-70 year olds, with 19% and 16% respectively (upper limits of 23% and 19%). Based on the referred people in these latter age groups, this would result in an adjusted coverage of approximately 30% and 24% (upper limits 34% and 29%) [Table 5].

Table 3 Prevalence rates for separate cardiovascular risk conditions and comparison with literature. Prevalence per 100 people with lower and upper limits and range over the age groups and sex distribution with peak prevalence.

Risk-conditions Prevalence/100	Overall	Range for age groups	Lower-upper limits	Male; peak	Female; peak	Range in literature	Lit ref #
Heart valve defect	2.39	0.1-17.6	2.23-2.56	2.14; 16.67	2.62; 18.01	0.2-13.0	28-33
Aortic aneurysm/ prosthesis	0.56	<0.05-3.0	0.49-0.65	0.89; 6.74	0,26; 3.39	up to 8.9	33-38
Congenital heart anomaly	0.36	<0.1-0.7	0.30-0.43	0.34; 0.55	0.37; 0.85	0.4-0.8	39-44
Endocarditis	0.11	0-0.28	0.08-0.16	0.10; 0.28	0.12; 0.35	0.12	45-46
Any risk condition	3.1	0.6-18.5	2-94-3.32	3.10; 19.00	3.15; 18.63	-	-

Table 4 Risk-conditions for chronic Q fever of screened patients in the vaccination campaign in 2011. Only patients included from the high-incidence area, stratified according to age groups and sex.

Risk-conditions and age groups	15-20	20-30	30-40	40-50	50-60	60-70	70-80	80-90	>90 yrs	all
Heart valve defect	14	14	20	62	145	305	280	85	4	929
Aortic aneurysm/prosthesis	0	1	1	4	33	101	122	32	0	294
Congenital heart anomaly	17	14	21	34	11	8	4	0	0	109
Endocarditis	3	0	3	4	9	10	7	1	0	37
Any risk-condition	30	30	40	97	192	417	409	111	4	1330
male	15	19	20	44	111	283	257	64	2	815
female	15	11	20	53	81	134	152	47	2	515

Table 5 Coverage of Q fever vaccination in the high-incidence area, for risk-conditions separately and combined. Coverage based on screened patients in the Q fever vaccination campaign from the high- incidence area and stratified for age groups and sex. Lower and upper limits based on 95%CI of the prevalence estimates. Adjusted coverage for all age groups combined calculated with the number of referred patients from the high-incidence area in the Q fever vaccination campaign.

Coverage and age groups	15-20	20-30	30-40	40-50	50-60	60-70	70-80	80-90	≥90yrs	all	lower limit	upper limit	(all adjusted)
Heart valve defect	30%	14%	13%	12%	21%	16%	11%	4%	1%	11%	10%	11%	(17%)
Aortic aneurysm / prosthesis	0%	8%	0%	5%	12%	19%	15%	6%	0%	12%	11%	14%	(21%)
Congenital heart anomaly	8%	5%	14%	13%	10%	5%	10%	0%	0%	9%	7%	11%	(16%)
Endocarditis	10%	0%	11%	28%	13%	10%	10%	8%	0%	12%	8%	18%	(27%)
Any risk condition	14%	9%	13%	12%	19%	16%	13%	5%	1%	11%	11%	12%	(18%)
lower limit	8%	6%	9%	9%	15%	14%	11%	4%	1%	-	-	-	-
upper limit	24%	13%	20%	15%	23%	19%	14%	5%	1%	-	-	-	-
male	19%	10%	17%	12%	21%	20%	15%	6%	2%	14%	13%	15%	(23%)
female	11%	7%	11%	12%	16%	12%	10%	4%	1%	9%	8%	10%	(15%)

Discussion

This study aimed to evaluate the quantitative performance of the Q fever vaccination campaign in 2011, which targeted people at risk for chronic Q fever. Prevalence data for the defined risk-conditions were not available at the time and no disease registers exist in the Netherlands. In this retrospective cohort study, we identified the number of people with those predefined risk-conditions for chronic Q fever in a random sample of a population-based database, from which we calculated stratified prevalence estimates. By extrapolation of these prevalence data to the population of the high-incidence area of the Q fever epidemic, we arrived at an approximation of about 12,000 people with a definite or probable cardiovascular condition predisposing to chronic Q fever for this particular area. Using the recorded number of people actually screened for Q fever vaccination from this area, we estimated the overall coverage to be 11% (upper limit 12%). The adjusted coverage estimate, based on the larger group of referred people, was 18%.

There are several possible explanations for this limited coverage. First, the centralized approach, with only one location and limited dates for screening and vaccination may have posed a barrier for eligible people. Moreover, potential vaccinees had to travel twice because of the required and invasive pre-vaccination skin test and serology. As the vaccine was unregistered, information leaflets and letters were sent to cardiologists and GPs and to potential vaccinees, in which uncertainties were described explicitly; this may have influenced their willingness to refer or to be vaccinated. Furthermore, potential vaccinees needed to sign multiple consent forms, which may have been another barrier to refer or to consent. The necessity to fill in several diaries to record experienced adverse events and the intensified safety surveillance with follow-up by telephone may have put off quite some referred people. Reasons for secondary withdrawal were, among others, too far- unsafe/perceived risk benefit misbalance- intercurrent illness- feeling to be a guinea pig- difficult/impossible day/date- lack of transport- etcetera. Some potential vaccinees could not walk, were bedridden, or felt they were not at risk because they hardly ever left their home. Some other people were excluded because one felt they were unable to comply with the requirements of informed consent and follow-up. Hence the low coverage in the oldest age groups. Moreover, when the vaccination campaign finally took off, the outbreak was already in decline due to the effects of consecutive veterinary interventions in the year before; the urgency of vaccination was felt less, not only by referring GPs and their patients but also by the coordination team of the campaign and its implementers. In December 2010, HC decided not to include (new) occupational risk groups because risk of infection had sufficiently decreased [18]. This led to the decision not to extend the relative short intake period for the Q fever vaccination campaign. In contrast with

population based campaigns or programmes, no repeated public education and information was launched. To avoid the so-called "worried well" phenomenon, the public were not addressed directly. The short time span between informing the public and professionals, recruitment of potential vaccinees and the screening and vaccination period, posed an obstacle to timely referral and intake. As reported [11], GPs had varying degrees of difficulty to identify eligible patients from the files depending on their file system specifications and codes. Lastly, the lack of prevalence data may have negatively influenced coverage in an indirect way, as inadequate logistics for coping with a much larger than expected number of indicated patients may have put off some patients, because convenient appointments could not easily be made.

For coverage estimation, we left out all cases labelled as possible from the IPCI-study population because many were early cases and of the mildest severity, without full clinical work up. GPs might not interpret most of these patients to be at high-risk for chronic Q fever; neither would these people think so themselves. We calculated coverage for people included in pre-vaccination screening, as this group was effectively enrolled, instead of for people referred as the larger group that was potentially reached. For this latter group, adjusted coverage would be approximately 50% higher. Coverage estimation was based on the Q fever high-incidence area (75% of all screened people), for which we had retrieved background population numbers.

The coverage estimates varied somewhat among risk-conditions and age groups. The low coverage in the two oldest age decades is not surprising in the light of the obstacles and criteria mentioned before. The highest coverage was in the (largest) middle age groups, with 19% and 16% for 50-60 and 60-70 years old, respectively. Men had higher coverage rates than women. In the light of the higher Q fever risk in these specific age groups and in men [19], this is a positive finding. These risk factors were included in the HC-advice [6] and in information letters to GPs, possibly leading to increased referral. Women were underrepresented in referral and screening for Q fever-vaccination. An explanation might be that they have milder (asymptomatic) cardiovascular defects or were less receptive to be referred for vaccination [20].

In contrast to the well-incorporated Dutch universal childhood vaccination programme (over 95% coverage, except for HPV due to concerns about safety and necessity) [21][, coverage for other (adult) risk-group vaccination is often disappointingly low, even after official and repeated recommendations [22-26]. The annual influenza vaccination programme sticks out comparatively positively but for the hepatitis B vaccination for men having sex with men the estimated annual coverage of only 1% [27]. Such risk-group targeted vaccinations are also hard to monitor, especially if executed in the private sector without disease databases and vaccination registers [28].

The calculated prevalence estimates for the four risk-conditions showed that overall, 3.1% of the population of 15 years and older had one or more of the defined conditions, increasing with age and similar for men and women. One might think that prevalence estimates could have been retrieved from literature before the start of the vaccination campaign. This could have prevented some of the logistic pressures because of the unexpected large influx of referrals. Data on prevalence of valvular defects [29-34], aortic aneurysms [35-39], congenital heart anomalies [40-45], and endocarditis [46,47] can indeed be found in literature [Table 3]. Some publications are quite recent, however, and very few are from the Netherlands [37]. There is no reason though, to expect substantial differences in prevalence rates between developed countries, but some racial or ethnic differences have been described [48], as well as changes over time, better therapeutic procedures and higher life expectancy [43].

The prevalence estimates in this study are the first available in the Netherlands. Heart valve defects, as the most common risk-condition presented in 2.4%, with peak prevalence of 17.6% in the older age groups. Aortic aneurysm/prosthesis occurred in 0.6%, with peak prevalence of 3%. Congenital cardiovascular anomalies were a rather small group in the IPCI-study population as well as in the Q fever vaccination campaign and were comparatively overrepresented in the youngest age group . This may be because patients with the more severe defects may not have survived, or recent severe degenerative or acquired symptomatic changes may have overshadowed the primary condition. Prevalence estimates for all risk-conditions seem to be well in line with the rather scarce reports in literature.

Observed prevalence rates indicate that a very large group of people in the population is at substantial risk of Q fever complications. Not only those living in the high-incidence area at the time, but also those living in the proximity of infected farms in other areas. Of note, environmental *Coxiella burnetii* has the potential to spread over great distances [49,50]. Seroprevalence studies conducted in 2009-2011 have found a large proportion (>10%) of people infected by *C .burnetii* and illustrates the magnitude of the Dutch Q fever outbreak [51]. For the future, containing Q fever is of great importance, and relaxation of veterinary measures, i.e. mandatory vaccination and tank milk surveillance for Q fever at goat-farms, could pose a great hazard for this prevalent risk group. Therefore, veterinary measures containing Q fever need to be continued.

A difficulty for this study was that the risk-conditions searched for in the IPCI-database required long string search terms in addition to ICPC-codes and manual validation. This was necessary because much information on these risk-conditions was captured in unstructured text in GP information systems. Medical expertise was necessary to validate the patients, because patient-records had to be assessed in medical perspective. With searches based on ICPC-codes alone, a large

proportion of qualifying patients would have been missed. Patients with only an ICPC-code for risk-condition, qualified for the lowest level of diagnostic certainty (possible) at most, because free text substantiation was missing. In addition, some patients with an incorrect code would have been included without the free text validation. Some miscoding may occur after several consecutive changes in the coding system and because of misunderstanding by the coding-assistant. For instance, several times, the rheumatic fever code -in Dutch acute rheuma(tic disease)- was mistakenly chosen in cases of acute presentation of polyarthritis rheumatica. Perusing the free text entries, this could be sorted out in the majority of cases. We may have missed less obvious misconceptions, however. In addition, not all risk-conditions or targeted interventions had codes that were specific enough.

As the Q fever vaccination campaign was not designed as a study, indications for referral, especially in people with multiple risk-conditions, may not have been completely reported. On the other hand, checking of people's eligibility was strict and all underlying risk- conditions were critically reviewed, before acceptation for pre-vaccination screening. Even so, only a small percentage (2.9%) of referred people was rejected because proper indication lacked or because of young age.

A strength of this study is that the IPCI-study population –which we used to calculate prevalence estimates of the risk-conditions– was representative of the Dutch population. The existence of population registers enabled us to extrapolate these prevalence estimates to the Q fever high-incidence area. The consistency of the yield with prevalence data available from literature and the similar relative frequencies for the respective risk-conditions in the Q fever vaccination campaign and the IPCI-study population inspire confidence. We assume high sensitivity of our search strategy with the broad search terms we chose (including spelling errors), while we did not come across other terms or synonyms for the conditions we were after in the free text of the validated records. In this perspective, the relative high proportion of rejections after validation was reassuring. We increased specificity through this individual validation of the full extracted patient group and subsequent review of all borderline cases with subsequent inclusion or rejection. Moreover, the called for risk-conditions had a relative high frequency in the general population resulting in precise estimates with small 95% confidence intervals.

In conclusion, the estimated coverage of the Q fever vaccination campaign of 11-18% was limited, but is understandable if all obstacles are taken into account. The IPCI-database extraction for determination of risk-condition prevalence rates turned out to be a feasible procedure within a limited time frame. It is fit for future use in case background rates are necessary for policymaking, implementation and surveillance of (public) health interventions, provided proper and specific (low

level) codes exist for disease (sub)groups. This may be particularly true for the planned efforts to launch recommendations on the benefit of vaccinations for subgroups in addition to universal vaccination programmes [52-54]. This holds even truer for those risk-conditions for which prevalence data are missing, and the size of the targeted subgroup needs to be estimated.

Acknowledgements
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Table S1 Risk-conditions for chronic Q fever in the IPCI-study population, according to age groups for cases with definite of probable diagnostic certainty. Distribution of the full IPCI-study population; number of risk-condition cases in the IPCI-study population; prevalence estimates of risk-conditions per 10,000 according to sex, as calculated from the IPCI-study population, with lower and upper limits.

IPCI-study population (number)	15-20	20-30	30-40	40-50	50-60	60-70	70-80	80-90	> 90 yrs	all
all	2158	4708	5001	6224	5509	4454	2837	1447	233	32571
male	1093	2354	2400	3047	2728	2138	1300	579	72	15711
female	1065	2354	2601	3177	2781	2316	1537	868	161	16860
male%	51%	50%	48%	49%	50%	48%	46%	40%	31%	48%
Risk-conditions (number def+prob)										
heart valve defect	3	8	11	35	49	134	193	159	38	630
aortic aneurysm/prosthesis	0	1	0	6	19	37	61	45	4	173
congenital heart anomaly	14	20	11	18	8	11	3	3	0	88
endocarditis	0	1	2	1	5	7	5	1	0	22
any risk condition	14	26	22	57	72	176	236	198	40	841
male	5	14	8	26	36	95	121	96	12	413
female	9	12	14	31	36	81	115	102	28	428

Prevalence of risk-conditions (def+prob) (/10,000)										
heart valve defect	14	17	22	56	89	301	680	1099	1631	193
male	9	21	17	46	77	318	662	1071	1528	173
female	19	13	27	66	101	285	696	1118	1677	212
aorta aneurysm/prosthesis	0	2	0	10	34	83	215	311	172	53
male	0	4	0	13	51	126	377	639	417	86
female	0	0	0	6	18	43	78	92	62	23
congenital heart anomaly	65	42	22	29	15	25	11	21	0	27
male	46	42	17	30	15	19	15	17	0	25
female	85	42	27	28	14	30	7	23	0	29
endocarditis	0	2	4	2	9	16	18	7	0	7
male	0	4	0	0	11	19	15	17	0	7
female	0	0	8	3	7	13	20	0	0	7
any risk-condition	65	55	44	92	131	395	832	1368	1717	258
lower limit	37	38	29	71	104	342	736	1201	1287	242
upper limit	109	81	67	119	164	456	939	1555	2253	276
male	46	59	33	85	132	444	931	1658	1667	263
female	85	51	54	98	129	350	748	1175	1739	254

Table S2 Estimated numbers of people with risk-conditions for chronic Q fever in the high-incidence area. Numbers shown per age group and sex based on the prevalence rates for cases with definite and probable diagnostic certainty from the IPCI-study population.

Eligible people high-incidence area (estimated numbers)	15-20	20-30	30-40	40-50	50-60	60-70	70-80	80-90	>90yrs	**all**
Heart valve defect	47	102	152	498	703	1940	2662	1921	392	8782
Lower limit	16	52	85	358	532	1642	2322	1657	292	8128
Upper limit	138	202	271	691	928	2291	3048	2220	519	9490
Aorta aneurysm/prosthesis	30	13	27	85	273	536	841	544	41	2412
Lower limit	0	2	0	39	175	389	657	408	16	2079
Upper limit	60	72	53	186	425	737	1077	723	104	2797
Congenital heart anomaly	219	256	152	256	115	159	41	36	24	1227
Lower limit	131	166	85	162	58	89	14	12	0	996
Upper limit	367	395	271	404	226	285	121	106	47	1511
Endocarditis	30	13	28	14	72	101	69	12	24[a]	307
Lower limit	0	2	8	3	31	49	29	2	0	203
Upper limit	60	72	100	81	168	209	161	68	47	464
Any risk condition	219	332	303	811	1033	2549	3255	2392	413	11724
Lower limit	125	227	200	626	821	2204	2879	2100	310	10965
Upper limit	367	486	459	1049	1299	2944	3675	2719	542	12532
Male	80	184	116	381	528	1446	1688	1043	89	5922
Lower limit	34	110	58	261	382	1188	1423	866	52	5384
Upper limit	186	309	228	557	728	1758	1996	1247	143	6513
Female	138	149	184	428	505	1117	1571	1315	326	5808
Lower limit	73	85	110	302	366	902	1317	1094	231	5289
Upper limit	261	260	309	606	698	1382	1870	1574	450	6376

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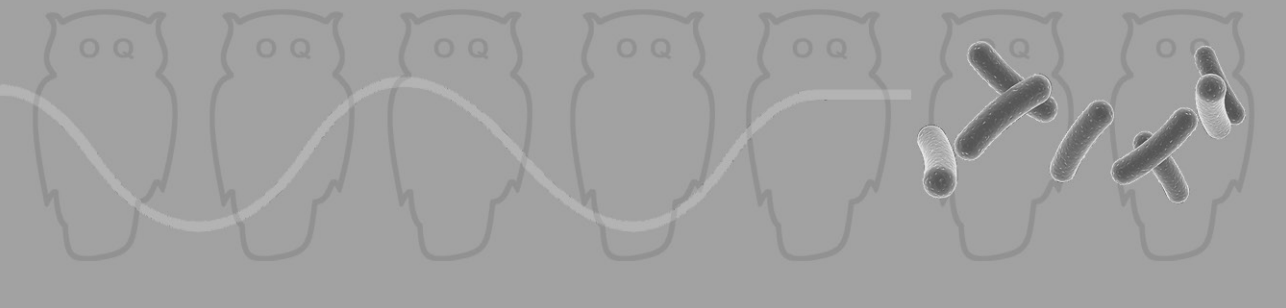
Summary and conclusions



The Q fever outbreak in the Netherlands was the largest ever reported, and afflicted many people, especially those suffering from chronic sequelae of Q fever infection. The outbreak, however, also offered the opportunity to gain new insights in the interaction between *C. burnetii* and the immune system of the human host. A better understanding of host's immune responses to *C. burnetii* infection and vaccination is important to improve prevention, diagnosis and treatment of the life-threatening disease chronic Q fever.

In this thesis, studies have been performed to investigate the immunological response to *C. burnetii* in healthy individuals and chronic Q fever patients, to develop a new diagnostic tool that adds in the diagnosis of Q fever, to assess the risk of immune-modulating drugs on the development chronic Q fever, to investigate the safety and immunogenicity of vaccination against *C. burnetii* in a highly relevant population of people at high-risk for chronic Q fever and to determine the coverage of this targeted vaccination campaign.

PART I. Studies on the immune response against *C. burnetii* in (chronic) Q fever

The innate immune system has an essential role in the host defence against invading *C. burnetii* through recognition of the bacterial components and production of cytokines to activate anti-microbial mechanisms. Two main pattern recognition receptors (PRRs) of the host's immune cells that recognize bacterial components are Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs) NOD1 and NOD2 (1,2). Several reports suggest that TLR4 in mice is redundant for anti-*C. burnetii* responses, as is NOD2, while TLR2 is involved in recognition and host responses to avirulent phase II bacteria; TLR2 is however dispensable for clearance of phase I *C. burnetii* in vivo in mice (3-7). Little is known about the role of the various PRR for recognition of *C. burnetii* in humans. In **Chapter 2**, we investigated the role of several TLRs and NOD1 and NOD2, and the downstream of TLRs mitogen-activated protein kinases (MAPKs) - p38, JNK, and ERK - in the recognition and cytokine response against two *C. burnetii* phase I strains: the reference strain *C. burnetii* Nine Mile and the Dutch outbreak isolate *C. burnetii* 3262. We found that TLR1, TLR2 and NOD2 are involved in the cytokine responses against *C. burnetii* in human peripheral blood mononuclear cells (PBMCs), while TLR4 and NOD1 are not. Inhibition of TLR2, p38, JNK and ERK led to decreased cytokine response in *C. burnetii*-stimulated human PBMCs. PBMCs of individuals bearing the *NOD2* 3020insC mutation, causing loss of function of NOD2, had significantly lower cytokine responses to *C. burnetii*. The same was observed in PBMCs of human donors with genetic polymorphisms in *TLR1* gene. The involvement of NOD2 and TLR1 in cytokine response to *C. burnetii* was

confirmed in experiments in which bone marrow-derived macrophages (BMDM) of NOD2 knockout mice and TLR1 knockout mice showed defective IL-6 production upon *C. burnetii* Nine Mile stimulation in vitro. Interestingly, in humans, TLR6 seemed to be involved in cytokine responses to the Dutch isolate 3262, but not to the Nine Mile strain, based on the effect of genetic polymorphisms in the *TLR6* gene on cytokine production. This needs further research, but might be explained by different binding sites of the isolate 3262 to TLR6 than the Nine Mile strain. *C. burnetii* 3262 was the predominant genotype found during the Q fever outbreak in the Netherlands (8). Based on the fact that it caused such an unprecedented outbreak, one can hypothesize that this strain is more virulent than other strains. It can be questioned whether extended activation of the immune response by TLR6 is beneficial for the infected individual, as enhanced uptake of *C. burnetii* by monocytes and macrophages might favour the intracellular replication of *C. burnetii*.

Inadequate early recognition and cytokine response to *C. burnetii* may result in inefficient early clearance and increased likelihood to develop chronic Q fever. Genetic variants in immune cell receptors such as TLRs and NODs and their adaptors have been associated with increased susceptibility to bacterial infections (9-13). No study so far has been able to investigate this for chronic *C. burnetii* infection, due to the low prevalence of the disease. In the aftermath of the acute Q fever epidemic, a large number of patients with chronic Q fever were observed. In **Chapter 3,** we therefore investigated whether genetic variants in PRRs and adaptor proteins are associated with chronic Q fever. Twenty-four single nucleotide polymorphisms (SNPs) in genes encoding TLR1, TLR2, TLR4, TLR6, NOD2, $\alpha_v\beta3$-integrin, CR3, and adaptors MyD88 and TIRAP were genotyped in 139 chronic Q fever patients and in 220 control individuals with cardiovascular risk-factors and previous exposure to *C. burnetii* without development of chronic Q fever. Associations between these SNPs and chronic Q fever were analyzed and cytokine production in whole blood stimulation assays was correlated to relevant genotypes. These analyses identified polymorphisms *TLR1* R80T, *NOD2* 1007fsX1 and *MYD88* -938C>A to be associated with development of chronic Q fever. Individuals carrying the *TLR1* 80R allele showed increased IL-10 production in whole blood upon *C. burnetii* exposure in vitro, which may contribute to the increased risk of chronic Q fever.

In **Chapter 4**, we investigated the IFN-γ response to *C. burnetii* in chronic Q fever patients. We found, in contrast to what has been generally assumed, that the IFN-γ response is not defective in chronic Q fever. We showed that immune cells of chronic Q fever patients are not only capable of a high IFN-γ response to *C. burnetii*, but also display up-regulation of genes downstream of the IFN-γ receptor in

addition to production of IFN-γ-dependent neopterin. We found that genetic polymorphisms in *IL12B* are associated with the development of chronic Q fever. Our findings can be linked to the observation that anti-*C. burnetii* antibody titers are high in chronic Q fever patients. Taken together, this strongly suggests that chronic Q fever patients do not have a defective adaptive immune response. More likely, the innate immune response, or the link between innate and adaptive immune response might be inadequate in these patients to clear the infection with *C. burnetii* in an early phase. We should, however, take into account that we studied the immune response of circulating blood mononuclear cells to *C. burnetii*. It may be more relevant to study the local immune response in *C. burnetii* infected vascular walls or valvular tissue. Both vascular and valvular chronic *C. burnetii* infection are mostly low-grade infections in which systemic immune activation is apparently not effective in clearing the local infection. The local immunological processes are crucial for survival of *C. burnetii* at predilection sites such as defective cardiac valves and aneurysms of the vascular wall. Previous immunohistochemical studies of *C. burnetii*-infected cardiac valves showed small, focal collections of infected mononuclear phagocytes (14,15). Characterization of these local immune cells and their immunological environment is subject to future research.

PART II. Detection of *C. burnetii* infection using cell-mediated immune responses

The standard immunological method to detect Q fever is measurement of specific antibodies against phase I and phase II *C. burnetii*. In part II of this thesis, we investigated the usefulness of an alternative diagnostic method based on cell-mediated immune responses – i.e., by measuring IFN-γ production in blood after in vitro exposure to *C. burnetii* antigens.

An extensive study was performed to investigate the accuracy of this assay in diagnosing previous *C. burnetii* infection (past Q fever); this study is described in **Chapter 5**. The in-vitro IFN-γ production assay was carried out in a group of 1,525 vaccination candidates. Different formats, using different *C. burnetii* antigens were tested, of which undiluted whole blood culture with Nine Mile antigens showed the best performance. All subjects were also screened by Q fever serology and skin-test, and these outcomes were related to the IFN-γ production. We performed Bayesian analysis that showed that the IFN-γ production assay had similar sensitivity and specificity as the combination of serology and skintest to detect previous infection with *C. burnetii*. Agreement between the tests was moderate; however, discordant results could not be resolved because there is no gold standard. We concluded that the IFN-γ production assay should be considered complimentary to Q fever serology.

In **Chapter 6** we investigated whether the measurement of IFN-γ production in *C. burnetii*-stimulated whole blood, in combination with other cytokines, could be used to differentiate a past (cleared) infection from an ongoing infection (chronic Q fever) with *C. burnetii*. We found that high IFN-γ in combination with low IL-2 production was very specific for chronic Q fever. The relevance of measuring IFN-γ/IL-2 production profiles as diagnostic correlate of T-cell responses, has been studied outside the field of Q fever. The IFN-γ/IL-2 ratio reflects the type of effector and memory CD4+ T-cell response (16). Memory T-lymphocytes can broadly be defined as two distinct populations of effector memory T-cells and central memory T-cells. IFN-γ is predominately produced by effector T-cells and effector memory T-cells, while IL-2 is predominately produced by central memory T-cells. It is postulated that high IFN-γ/IL-2 ratio indicates predominance of effector T-cells and effector memory T-cells cells, resulting from ongoing immunological stimulation by a persistent infection. We concluded that the IFN-γ/IL-2 ratio could be used as an additional diagnostic marker in the timely diagnosis and early treatment of chronic Q fever to prevent severe morbidity and death.

In **Chapter 7** we performed a longitudinal study to assess the applicability of the measurement of IFN-γ/IL-2 ratio in the follow-up during treatment of chronic Q fever patients. The current clinical, imaging and microbiological parameters to monitor the response to treatment are based on experts opinion and small-sized retrospective studies (17, 18), and clinicians experience difficulties in decision making about when antibiotic treatment can be discontinued. Thus, there is a need for additional biomarkers that correlate with treatment success and clearance of infection.

We measured the *C. burnetii*-specific IFN-γ and IL-2 production in a whole-blood stimulation assay during a period of at least 18 months follow-up of 15 proven chronic Q fever patients. We observed a trend in which the IFN-γ/IL-2 ratio declined when patients experienced a successful outcome of treatment. Patients in whom treatment failed, had overall lower IFN-γ/IL-2 ratios, which did not significantly decrease. Previous studies in viral infections and tuberculosis have correlated the T-cell IFN-γ and IL-2 production to antigen load (19); these studies showed that clearance of infection with decreasing antigen load coincides with a shift from predominantly IFN-γ secreting CD4+ T-cells to IL-2 secreting CD4+ T-cells. Our finding that the IFN-γ/IL-2 ratio declines during successful treatment of chronic Q fever, assuming decrease in antigen load, is in accordance with these studies. Patients with unsuccessful treatment show lower fluctuating IFN-γ/IL-2 ratios from the start. They might have bacterial persistence with low antigen concentrations, an assumption that is supported by the notion that in most of these patients, *C. burnetii* DNA was not detectable in blood even before start of antimicrobial therapy.

Another IFN-γ production assays has been developed during the Dutch Q fever epidemic, which is based on a technique of enzyme-linked immunosorbent spotting (Elispot); here antigen-specific IFN-γ secreting cells are detected by making them visible at a single-cell level (20). In **Chapter 8**, we compared the results of our IFN-γ production assay with the results of a *Coxiella* Elispot in a group of 16 chronic Q fever patients and 17 healthy control individuals. We found that these techniques had similar high sensitivity (14/16, 88%) to discriminate patients from controls, but the Coxiella Elispot seemed to be more specific than the IFN-γ production assay when Nine Mile was used as stimulating antigen. Interestingly, the quantitative outcomes of both assays showed moderate correlation. The discrepancies can be explained by the different origins and concentrations of stimulating antigens, the different phase variation, or the method of inactivation of the antigen stimuli. Moreover, it may well be true that the amount of IFN-γ produced and released is not directly related to the number of IFN-γ positive T-cells.

The observations in this study, and in our previous studies of IFN-γ production assays in *C. burnetii* infection, show the potential of measuring cell-mediated immune responses for the diagnosis of past or chronic Q fever. IFN-γ based assays are already routinely used for detection of immunity to Mycobacterium tuberculosis and commercial kits are available for the two types of assays, e.g. the QuantiFERON-TB and the T Spot TB (21). These assays have been reported to be at least as accurate as the tuberculin skin test to detect exposure to *M. tuberculosis*.

In **Chapter 9** we wanted to investigate the timing and extent of *Coxiella*-specific IFN-γ responses in an acute *C. burnetii* infection (acute Q fever). For this purpose, we used a model in which immunocompetent BALB/c mice were challenged with aerosols containing virulent phase I *C. burnetii*. The production of other cytokines was also measured in an effort to identify other potential diagnostic markers for acute Q fever. Whole blood incubation proved to be cumbersome in mice; as an alternative, splenocyte cultures were used for measurement of *C. burnetii* induced cytokine responses. This model demonstrated that *C. burnetii*-induced IFN-γ could be used to detect acute *C. burnetii* infection in mice, and this is likely to be the case in humans as well. Antigen-specific production of IFN-γ and IFN-γ-induced protein-10 (IP-10) were both detectable prior to elevation of specific IgG antibodies. This study also showed antigen-specific induction of IL-6, keratinocyte-derived cytokine (KC), and monocyte chemotactic protein-1 (MCP-1) from splenocyte cultures.

The central position of serology in Q fever diagnostic is undisputable, and serology has hitherto been the most widely used immunological measurement for *C. burnetii*

infection. It continues to be extensively validated in Q fever research (22-25). In the light of waning antibody titres years after infection, and the ongoing debate of cutoff values of serology for diagnosis of chronic Q fever, and the meaning of persistently high antibody titers without clinical signs or symptoms of chronic Q fever, we conclude that it would be valuable to take into account the more relevant cell-mediated immune response as an additional diagnostic tool.

PART III. Immunosuppressive drugs for rheumatoid arthritis as a risk factor for chronic Q fever

Immune suppression is a stated risk factor for development of chronic Q fever, which is based on animal studies and on limited human case series. The type of immune suppression that increases the risk for chronic Q fever has not been clearly defined. We hypothesized that the use of TNF-blockers in patients with rheumatoid arthritis (RA) or other inflammatory conditions might specifically increase the risk for chronic Q fever, as it does for other infections with intracellular bacteria such as M. tuberculosis. In **Chapter 10** we describe a study to the prevalence of anti-*C. burnetii* antibodies in RA patients with and without anti-TNF therapy living in the Q fever outbreak area. We identified cases of chronic Q fever in these cohorts. We found a similar prevalence of *C. burnetii* infection in RA patients with and without TNF-blockers. In the group of patients with TNF-blockers, 12.3% (7/57) of those with anti-*C. burnetii* antibodies were diagnosed with chronic Q fever, compared to 5.5% (3/55) in the anti-TNF naïve patient group (P=0.32). With this small number of cases, and consequently limited power, only a large effect (>four-fold) of anti-TNF therapy on the risk for chronic Q fever could have been detected. Interestingly, univariate analysis in all *C. burnetii*-infected RA patients (n=102), suggested that corticosteroid use was associated with development of chronic Q fever. Multivariate analysis could not be performed, due to low number of cases. Of note, the observed prevalence of chronic Q fever in *C. burnetii*-infected RA patients was considerably higher (8,9%) than previously reported in non-selected infected individuals in the same area (1,6%) (23). This indicates that RA and its treatment, either with or without anti-TNF, may be considered as a risk factor for the development of chronic Q fever. Therefore, we advise that in case of *C. burnetii* infection, all patients with RA should be carefully monitored for progression to chronic Q fever.

In **Chapter 11** we present a case history of a RA patient with acute Q fever while being treated with anti-TNF agents, who developed chronic Q fever while being switched to treatment with the anti-B-cell (anti-CD20) monoclonal antibody rituximab. We observed in this patient that the use of anti-TNFα agents for RA in the acute phase of Q fever does not seem to impede the *C. burnetii*-specific serological response. However an intact humoral response did not prevent

progression to chronic *C. burnetii* infection, most likely because essential cellular immune responses were suppressed in the acute phase of the infection. Even though anti-B-cell therapy with rituximab was started after the acute Q fever episode, an increase in anti-*C. burnetii* phase I antibodies was observed, leading to the diagnosis of chronic Q fever. We assume that *C. burnetii*-specific CD20-negative memory B-cells are responsible for this rise in antibody titres.

It is tempting to speculate that B-cell depleting medication during the first contact with neo-antigens of *C. burnetii* would have seriously hampered the development of any antibody response and the diagnosis of Q fever based on serological titers. In this case, we did not perform IFN-γ production measurements in whole blood stimulated with *C. burnetii*.

PART IV. Prevention of Q fever by vaccination against *C. burnetii*

Vaccination against *C. burnetii* can be performed to protect people against Q fever. There is one vaccine for human use available worldwide, Q-vax, which is an inactivated whole-cell vaccine.

According to the manufacturer's protocol, pre-vaccination Q fever screening with skin-testing and serology should be performed to exclude people with past Q fever who are presumed to have an increased risk for immune-mediated adverse events following vaccination. In the Dutch Q fever outbreak, the authorities decided to vaccinate people with cardiac valvular/vascular risk factors for chronic Q fever living in the Q fever epidemic area. This was a unique decision; *C. burnetii* vaccination has been previously only used in people at occupational risk for *C. burnetii* infection, which comprises an overall younger and healthier population.

In **Chapter 12,** we describe the safety and adverse events (AE) after Q fever skin test and vaccination in the Dutch Q fever vaccination campaign. Vaccination of 1370 patients did not reveal unexpected AE; however, 80% of vaccinees reported local AE, of which 26% were pronounced or extensive. Two causally-related serious AE (SAE; 0.1%) were reported, both concerning a persistent subcutaneous injection site mass, with no signs of abscess or granuloma formation. Although we cannot conclude with certainty, it seems that these were not due to immunological responses to the vaccine; rather they were the effect of deposition of the vaccine too deep above the tendon insertion area of the M. deltoideus. The occurrence of local AE after vaccination was associated with occurrence of local AE after skin-test (with negative skin test reading at day 7) and high IFN-γ production pre-vaccination.

Vaccinees with local AE after skin-test or after vaccination appear to have more pronounced post-vaccination immune responses, based on Q fever serology and IFN-γ production assay results 6 months after vaccination. We conclude from the data collected and analyzed in this study that Q fever vaccination was safe but

reactogenic in the high-risk population with valvular/vascular risk factors for chronic Q fever. Rates of local AE were higher in women, younger age groups and in those with positive immunological parameters before vaccination.

In **Chapter 13**, we assessed in a random sample of the 260 vaccinees, the post-vaccination immune responses to *C. burnetii*, 6 and 12 months after vaccination. At 6 months, we found that 46% of vaccinees showed low anti-*C. burnetii* antibody titres and 67% had a positive IFN-γ production assay result. At twelve months, both were 60%. For comparison, serological results of 200 individuals with a natural Q fever infection were positive in 99.5% at 6 and 12 months after infection, with relatively higher antibody titres. Thus, the immune response after Q-vax vaccination is lower and restricted to a smaller proportion than found after a natural Q fever infection. Previous studies of on immune responses after vaccination in Australian abattoir employees, farmers and veterinarians reported higher rates of seroconversion and cellular immune responses after vaccination (26-28), suggesting decreased vaccine immunogenicity in our older population with risk factors for chronic Q fever.

Interestingly, in this study we observed that vaccinees with a positive outcome in the IFN-γ assay pre-vaccination showed a higher seroconversion rate than IFN-γ negative vaccinees: 74% vs. 41% ($P<0.001$). We conclude from this that a positive IFN-γ assay before vaccination in seronegative vaccinees likely points to pre-existing immunity resulting in boosting by vaccination.

Chapter 14 focused on the skin test, which, as an in-vivo intracutaneous test, can elicit an immune response itself. We showed that the intracutaneous Q fever test, containing a low dose of Q-vax antigen, induces clear *C. burnetii*-specific immune responses in about half of the tested individuals with a positive Q fever skin test. In addition, a median 2-fold increase in antibody titre was found in patients with positive serology in pre-vaccination screening. Since all individuals in this study had evidence of pre-existing immunity (either a positive skin test or positive serology), this immune response should be considered as a boosting effect of the skin test. A similar boosting effect is described for the tuberculin skin test (TST) on subsequent interferon-γ release assay (IGRA) responses; these tests are often used in combination to diagnose latent tuberculosis (29). This phenomenon makes it difficult for clinicians to discriminate between boosting and conversion of any increase in the IGRA response after TST. Likewise, clinicians and epidemiologist in the field of Q fever should be aware of the phenomenon that individuals who have had a skin test, as is the current practice in Q fever pre-vaccination screening, may develop higher anti-*C. burnetii* antibody titres and a more robust in-vitro IFN-γ release in follow-up measurements.

In **Chapter 15,** we aimed to evaluate the quantitative performance of the Q fever vaccination campaign in the Netherlands in 2011, which targeted all people at risk for chronic Q fever living in the Q fever epidemic area. Prevalence data for the defined risk-conditions were not available at the time and no disease registers exist in the Netherlands. As a consequence, it was unclear before and after the vaccination campaign, what percentage of the target group had been vaccinated against Q fever. In this retrospective cohort study, we set out to estimate the coverage of the vaccination. We first identified the number of people with the predefined risk conditions for chronic Q fever in a random sample of a population-based database, from which we calculated the stratified prevalence estimates. After that, we extrapolated these prevalence data to the population of the high-incidence area of the Q fever epidemic, and arrived at an approximation of about 12,000 people with a definite or probable cardiovascular condition predisposing to chronic Q fever for this particular area. Using the recorded number of people actually screened for Q fever vaccination from this area, we estimated the overall coverage to be 11%. The adjusted coverage estimate, based on the larger group of referred people, was 18%. This low estimated coverage should be interpreted in the light of the complexity of this target-group with much co-morbidity, and of the vaccination procedure that required two visits to a municipal health clinic with an invasive pre-vaccination screening. In addition, it is known that coverage for other adult risk-group vaccination is often disappointingly low, even after official and repeated recommendations. For example, the hepatitis B vaccination for men having sex with men has an estimated annual coverage of only 1% in the Netherlands (30). Since the Q fever epidemic waned in subsequent years, the Q fever vaccination was limited to January-April 2011, and was not continued in the years that followed.

Future perspectives

The studies in this thesis have given us new insights in the immunology of Q fever, but many questions are still unanswered and even more new questions have arisen. Our future research shall mainly focus on deciphering the innate immune response of the host against *C. burnetii*, and find out which factors predispose individuals to inefficient clearance of the infection leading to a chronic infection. Even though we found that IFN-γ is not deficient in chronic Q fever patients, we wonder whether patients that do not improve during long-term antibiotic treatment would benefit from adjunctive immune-stimulatory therapy with IFN-γ at pharmacologic dosages. Enhancing the host defence mechanisms by activating mononuclear phagocytes, might be beneficial to chronic Q fever patients with low IFN-γ production to *C. burnetii* in vitro in the first place.

Another important question to be answered is how *C. burnetii* is able to survive inside mononuclear cells at predilection sites such as cardiac valve tissues and aneurysm vascular wall. Better understanding the local immunological environment in aneurysms infected by *C. burnetii* and the mechanisms involved in damaging of the tissue might be the key to better treatment options for chronic Q fever patients. In conclusion, by increasing our knowledge on immunological aspects of Q fever, we can improve the care for patients suffering from this infectious disease.

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Samenvatting en conclusies

De grote Q-koorts epidemie in Nederland tussen 2007 en 2010 heeft veel mensen getroffen, van wie velen nu nog steeds klachten ondervinden. Deze uitbraak heeft echter ook de mogelijkheid geboden tot wetenschappelijk onderzoek naar de interactie tussen de Q-koorts bacterie - *Coxiella burnetii* - en het menselijk afweersysteem. Meer begrip over deze zogenaamde 'immunologische' aspecten van Q-koorts zal bijdragen aan betere mogelijkheden om Q-koorts te voorkomen, te diagnosticeren en effectief te behandelen.

Dit proefschrift beschrijft een aantal studies naar de immunologie van *C. burnetii* infectie in het licht van chronische Q-koorts en Q-koorts vaccinatie. In **deel I** bestuderen we de immuun respons tegen *C. burnetii* bij gezonde vrijwilligers en Q-koorts patiënten. In **deel II** beschrijven we een nieuwe diagnostische test voor Q-koorts, die gebaseerd is op immunologische methoden. In **deel III** onderzoeken we het verband tussen het gebruik van immunomodulerende geneesmiddelen bij reumatoïde artritis en de ontwikkeling van chronische Q-koorts. In **deel IV** beschrijven we de veiligheid en immunogeniciteit van vaccinatie tegen *C. burnetii* en de dekkingsgraad van de Nederlandse Q-koorts vaccinatie campagne.

Hoofdstuk 1 is een algemene inleiding over Q-koorts met de nadruk op immunologische aspecten van *C. burnetii* infectie. Tevens wordt hierin een overzicht gegeven van de inhoud en de doelen van dit proefschrift.

DEEL I. Studies naar de immuun respons tegen *C. burnetii* bij (chronische) Q-koorts

Het aangeboren immuun systeem beschermt de gastheer tegen *C. burnetii* door herkenning van onderdelen van de bacterie en productie van signaalstoffen – cytokines – , waardoor antimicrobiële mechanismen in werking worden gezet. De bacteriële componenten worden o.a. herkend door – op immuun cellen aanwezige – Toll-like receptoren (TLRs) en nucleotide-binding oligomerization domain-like receptoren (NLRs; NOD1 en NOD2) [1,2]. Eerdere studies hebben laten zien dat TLR4 bij muizen geen rol van betekenis speelt in de afweer-reactie tegen *C. burnetii*. Hetzelfde geldt voor NOD2. Daarentegen zou TLR2 betrokken zijn bij de herkenning en immuun respons tegen de avirulente *C. burnetii* fase II, terwijl er voor het opruimen van de virulente *C. burnetii* fase I geen tussenkomst van TLR2 is aangetoond in muisexperimenten [3-7]. Het is nog niet goed bekend of in menselijke afweercellen deze receptoren betrokken zijn bij de herkenning van *C. burnetii*.

In **hoofdstuk 2** onderzochten we de rol van verschillende TLRs, NLRs en van de adapter moleculen p38, JNK, en ERK, bij de cytokine respons op *C. burnetii* van humane perifeer bloed mononucleaire cellen (PBMCs). Hiervoor gebruikten we twee verschillende *C. burnetii* stammen: De laboratorium stam 'Nine Mile' (de

referentie stam) en de '3262' stam, de Nederlandse uitbraak stam. We vonden dat TLR1, TLR2 en NOD2 zijn betrokken bij de cytokine respons tegen *C. burnetii* door PBMCs, en TLR4 en NOD1 niet. Blokkade van TLR2, p38, JNK en ERK leidde tot een verminderde cytokine respons in *C. burnetii*-gestimuleerde humane PBMCs. PBMCs van individuen met een *NOD2* 3020insC mutatie, waardoor NOD2 niet meer werkt, hadden een significante verminderde cytokine respons tegen *C. burnetii*. Dit laatste werd ook gezien in PBMCs van gezonde vrijwilligers met genetische polymorfismen in het *TLR1* gen. De betrokkenheid van NOD2 en TLR1 werd bevestigd in experimenten met macrofagen verkregen uit beenmerg van muizen. Macrofagen van NOD2 knock-out muizen en TLR1 knock-out muizen produceerden minder interleukine-6 (IL-6) dan macrofagen van wildtype muizen, wanneer deze *in vitro* werden geïncubeerd met *C. burnetii*. Verrassend genoeg lijkt het erop dat bij mensen TLR6 is betrokken bij de cytokine respons tegen *C. burnetii* 3262, maar niet tegen *C. burnetii* Nine Mile, gebaseerd op het verschil in effect van genetische polymorfisme in het *TLR6* gen dat we observeerden tussen beide stammen. Dit moet verder worden onderzocht, maar een verklaring zou kunnen zijn dat TLR6 verschillende bindingsplekken heeft voor 3262 en Nine Mile. *C. burnetii* 3262 was de overheersende stam die werd geïsoleerd tijdens de Nederlandse Q-koorts uitbraak [8]. Dat deze stam in staat was om een uitbraak van dergelijke omvang te veroorzaken, doet vermoeden dat deze stam virulenter is dan andere stammen. De vraag is of een overmatige immuun respons via TLR6 gunstig is voor de gastheer, wanneer er meer *C. burnetii* in monocyten en macrofagen wordt opgenomen, of dat er daardoor mogelijk een voordeel is voor *C. burnetii* voor intracellulaire vermenigvuldiging.

Wij veronderstelden dat een inadequate vroege herkenning van *C. burnetii* bijdraagt aan het risico op de ontwikkeling van een persisterende infectie met *C. burnetii*, oftewel chronische Q-koorts. Eerdere studies naar andere bacteriële- en schimmelinfecties hebben aangetoond dat genetische variatie in TLRs en NLRs en bijbehorende adaptors geassocieerd is met verschil in vatbaarheid voor de desbetreffende infecties en de klinische uitkomst ervan [9-13]. Tot op heden is er nog geen studie verricht die een dergelijk verband voor chronische Q-koorts onderzocht, mede vanwege de lage prevalentie van de ziekte. In de nasleep van de Nederlandse Q-koorts uitbraak waren er veel chronische Q-koorts patiënten, waardoor deze studie mogelijk was. In **hoofdstuk 3** beschrijven we de resultaten van onze studie naar de associatie tussen genetische variaties in TLRs, NLRs en adaptor moleculen en de ontwikkeling van chronische Q-koorts. In deze studie werden 24 single nucleotide polymorphisms (SNPs) in de genen voor TLR1, TLR2, TLR4, TLR6, NOD2, $\alpha_v\beta 3$-integrin, CR3, en de adaptors MyD88 en TIRAP getypeerd bij 139 chronische Q-koorts patiënten en bij 220 controle individuen

met cardiovasculaire risicofactoren voor chronische Q-koorts en blootstelling aan *C. burnetii* echter zonder ontwikkeling van chronische Q-koorts. De associatie tussen deze SNPs en chronische Q-koorts werd geanalyseerd en de cytokine productie in volbloed stimulatie assays werd gecorreleerd aan de relevante genotypes. Dit toonde aan dat de polymorfismen *TLR1* R80T, *NOD2* 1007fsX1 and *MYD88* -938C>A geassocieerd zijn met chronische Q-koorts. In het bloed van individuen met het *TLR1* 80R risico allel werd *in vitro* na toevoegen van *C. burnetii* een verhoogde productie van het anti-inflammatoire cytokine IL-10 gevonden, wat kan bijdragen aan het risico op een persisterende infectie.

In **hoofdstuk 4** onderzochten we bij chronische Q-koorts patiënten de IFN-γ respons van hun immuun cellen op *C. burnetii*. We vonden, in tegenstelling tot wat algemeen werd aangenomen, dat de IFN-γ respons niet defect is in chronische Q-koorts. We toonden aan dat immuun cellen van chronische Q-koorts patiënten niet alleen in staat zijn tot een heftigere IFN-γ respons op *C. burnetii*, maar dat er tevens een toegenomen afschrift is van genen stroomafwaarts van de IFN-γ receptor, en dat een verhoogde productie plaatsvindt van neopterine, dat IFN-γ afhankelijk is. We vonden dat genetische polymorfismen in *IL12B* geassocieerd zijn met de ontwikkeling van chronische Q-koorts. Deze bevindingen gaan samen met de observatie dat chronische Q-koorts patiënten hoge anti-*C. burnetii* antistof titers hebben. Dit suggereert dat chronische Q-koorts patiënten niet een defecte verworven immuun respons hebben. Het is waarschijnlijker dat de aangeboren immuun respons, of de verbinding tussen de aangeboren en de verworven immuun respons, bij deze patiënten niet effectief is om de infectie met *C. burnetii* in een vroege fase te bestrijden. Als kanttekening moet worden geplaatst dat we de immuun respons van circulerende mononucleaire cellen op *C. burnetii* hebben bestudeerd. Het zou relevant zijn om de locale immuun respons tegen *C. burnetii* te bestuderen in geïnfecteerd vaatweefsel of hartkleppen. Zowel vasculaire als valvulaire chronische *C. burnetii* infecties zijn laaggradige infecties waarbij systemische immunologische activatie klaarblijkelijk niet in staat is om de lokale infectie effectief te bestrijden. De lokale immunologische processen zijn cruciaal voor de overleving van *C. burnetii* ter plaatse. Eerdere studies waarin immunohistochemie van *C. burnetii*-geïnfecteerde hartkleppen is verricht, lieten focale ophopingen zien van geïnfecteerde mononucleaire cellen [14, 15]. Toekomstig onderzoek zal zich richten op het karakteriseren van deze immuun cellen en de immuun respons ter plaatse in geïnfecteerd weefsel.

DEEL II. Diagnostiek naar Q-koorts: detectie van cellulaire immuun respons tegen *C. burnetii*

Serologie is de huidige standaard immunologische test voor Q-koorts; dit houdt in dat antistoffen tegen fase I en fase II *C. burnetii* worden gemeten. In deel II van dit proefschrift onderzoeken we een alternatieve diagnostische methode voor Q-koorts. Deze methode is gebaseerd op de cellulaire immuun respons, waarbij IFN-γ productie in bloed wordt gemeten na *in-vitro* incubatie met *C. burnetii* antigenen.

Een uitgebreide studie werd verricht naar de nauwkeurigheid van deze methode bij het vaststellen van doorgemaakte Q-koorts. Deze studie is beschreven in **hoofdstuk 5**. De *in-vitro* IFN-γ productie meting ("IFN-γ test") werd uitgevoerd in een groep van 1525 vaccinatie kandidaten. Alle deelnemers werden ook gescreend op Q-koorts door middel van serologie en een huidtest, waarvan de uitkomst werd vergeleken met die van de IFN-γ test. De IFN-γ test werd op verschillende manieren uitgevoerd. Het gebruik van onverdund volbloed geïncubeerd met fase I *C. burnetii* Nine Mile, liet de hoogste nauwkeurigheid zien. Bayesiaanse analyse toonde aan dat de IFN-γ test in deze vorm een sensitiviteit en specificiteit heeft die vergelijkbaar zijn met de combinatie van serologie en de huidtest, om een doorgemaakte Q-koorts aan te tonen. De overeenkomst tussen de uitkomsten van de testen was matig. Echter, disconcordante uitkomsten konden niet verder worden opgelost bij gebrek aan een gouden standaard.

In **hoofdstuk 6** onderzochten we of het meten van *in-vitro* IFN-γ productie in *C. burnetii*-gestimuleerd volbloed, in combinatie met het meten van andere cytokines, kan differentiëren tussen doorgemaakte Q-koorts (waarbij geen persisterende infectie is opgetreden) en chronische Q-koorts. We vonden dat een hoge IFN-γ productie in combinatie met een lage IL-2 productie heel specifiek is voor chronische Q-koorts. De relevantie van het meten van het IFN-γ/IL-2 productie profiel als weerspiegeling van het type T-cel respons is beschreven buiten het veld van Q-koorts. De IFN-γ/IL-2 ratio correleert met het type effector en memory CD4+ T-cel respons [16]. Memory T-cel lymfocyten worden onderscheiden in twee populaties: effector memory T-cellen en central memory T-cellen. IFN-γ wordt vooral geproduceerd door effector T-cellen en effector memory T-cellen, terwijl IL-2 vooral wordt geproduceerd door central memory T-cellen. Algemeen wordt aangenomen dat een hoge IFN-γ/IL-2 ratio duidt op dominantie van effector T-cellen en effector memory T-cellen, veroorzaakt door aanhoudende immunologische stimulatie door een persisterende infectie. Een lage IFN-γ/IL-2 ratio wijst op activatie van central memory T-cellen die aanwezig zijn na een geklaarde infectie. In lijn met deze bevindingen, vonden wij in onze studie dat chronische

Q-koorts patiënten zich onderscheiden van individuen met een doorgemaakte Q-koorts door een hoge IFN-γ/IL-2 ratio, met een specificiteit van 96%. We concludeerden dat de *C. burnetii* geïnduceerde IFN-γ/IL-2 ratio gebruikt kan worden als additionele marker voor de diagnostiek van chronische Q-koorts. Tijdige detectie en behandeling van chronische Q-koorts kan ernstige morbiditeit en overlijden voorkomen.

In **hoofdstuk 7** hebben we een longitudinale studie verricht om de bruikbaarheid vast te stellen van het meten van de *in-vitro* IFN-γ/IL-2 ratio gedurende de behandeling en follow-up van chronische Q-koorts patiënten. De huidige klinische en microbiologische parameters voor het monitoren van het effect van behandeling zijn gebaseerd op kleine retrospectieve studies en op adviezen van experts [17,18]. Vanwege het gebrek aan betrouwbare parameters is het voor behandelend artsen moeilijk om te beslissen wanneer antibiotische behandeling kan worden gestopt. Er is dus behoefte aan nieuwe biomarkers die correleren met het behandelsucces en het klaren van de infectie.

In deze studie hebben we *C. burnetii*-specifieke IFN-γ en IL-2 productie gemeten in een volbloed stimulatie assay gedurende een follow-up van tenminste 18 maanden van 15 bewezen chronische Q-koorts patiënten. Er was een duidelijke trend te zien waarin de IFN-γ/IL-2 ratio afnam bij patiënten met een succesvolle uitkomst van de behandeling. Patiënten bij wie de behandeling faalde, hadden over het algemeen aanvankelijk al een lagere IFN-γ/IL-2 ratio die niet verder afnam gedurende behandeling. Eerdere studies naar virale infecties en tuberculose hebben de correlatie onderzocht tussen de T-cell IFN-γ en IL-2 productie en de antigeen last [19]; deze studies hebben laten zien dat klaring van een infectie, gepaard gaande met een vermindering van de antigeen last, leidt tot een verschuiving van de aanwezigheid van hoofdzakelijk IFN-γ producerende CD4+ T-cellen naar IL-2 producerende CD4+ T-cellen. Onze bevinding dat de IFN-γ/IL-2 ratio afneemt gedurende succesvolle behandeling van chronische Q-koorts, met daarbij een aannemelijke afname van de antigeen last, komt daarmee overeen. Patiënten met falende therapie hadden zoals gezegd wisselend lage IFN-γ/IL-2 ratio's. Bij hen is er blijkbaar sprake van een persisterende infectie met een lage antigeen last. Deze veronderstelling wordt ondersteund door het feit dat juist bij de meeste van deze patiënten de PCR voor *C. burnetii* DNA in bloed negatief is, zelfs vóór start van antimicrobiële therapie.

Een andere IFN-γ specifieke test voor Q-koorts die tijdens de Nederlandse Q-koorts epidemie is ontwikkeld, is gebaseerd is op de techniek van enzyme-linked immunosorbent spotting (*Coxiella* Elispot). In deze test worden antigeen-specifieke IFN-γ producerende immuun cellen gedetecteerd op single-cell niveau [20]. In

hoofdstuk 8 hebben we de resultaten van de IFN-γ test vergeleken met die van de *Coxiella* Elispot in een groep van 16 chronische Q-koorts patiënten en 17 gezonde controle individuen. In deze groepen hadden de technieken een vergelijkbare hoge sensitiviteit (14/16, 88%) voor het onderscheid tussen patiënten en controle inidividuen, maar de *Coxiella* Elispot leek iets specifieker dan de IFN-γ test wanneer Nine Mile werd gebruikt als antigeen. Opvallend was dat de uitkomsten van de testen een matige correlatie lieten zien. De discrepantie kan worden verklaard door verschil in herkomst en concentratie van de antigenen, het fase verschil van het *Coxiella* lipopolysacharide, en het verschil in methode van inactivatie van de bacteriën. Bovendien is het zeer aannemelijk dat de hoogte van de IFN-γ productie en afgifte van IFN-γ niet direct correleert met de hoeveelheid IFN-γ positieve T-cellen.

De hiervóór beschreven studies met betrekking tot de IFN-γ productie test voor het aantonen van *C. burnetii* infectie, laten de potentie zien van het meten van de cellulaire immuun respons voor de diagnose van doorgemaakte en chronische Q-koorts. IFN-γ specifieke testen worden reeds routinematig gebruikt voor het aantonen van de immuun respons tegen *Mycobacterium tuberculosis*. Er zijn commerciële tests beschikbaar, de QuantiFERON-TB and the T Spot TB [21], die in studies minstens zo accuraat blijken te zijn als de tuberculine huidtest ("Mantoux test") voor het aantonen van latente tuberculose.

In **hoofdstuk 9** onderzochten we de timing en de hoogte van de *C. burnetii*-specifieke IFN-γ productie bij een acute *C. burnetii* infectie (acute Q-fever). Hiervoor gebruikten we een muismodel waarin immunocompetente BALB/c muizen werden blootgesteld aan aerosolen met virulente fase I *C. burnetii*. De productie van andere cytokines werd ook gemeten om andere potentiële diagnostische markers voor acute Q-koorts vast te stellen. Omdat volbloed incubatie bij muizen lastig bleek te zijn, werd gekozen voor *in-vitro* incubatie van splenocyten om *C. burnetii*-geïnduceerde cytokine productie te meten. Dit model liet zien dat *C. burnetii*-specifieke IFN-γ productie gebruikt kan worden om acute Q-koorts aan te tonen bij muizen. Het is aannemelijk dat dit ook bij mensen het geval is. *C. burnetii*-specifieke IFN-γ en IFN-γ geïnduceerd proteine-10 (IP-10) waren verhoogd voordat IgG-specifieke antistoffen aantoonbaar waren. Deze studie liet in splenocyten van *C. burnetii*-geïnfecteerde muizen ook *C. burnetii*-specifieke inductie zien van IL-6, evenals keratinocyte-derived cytokine (KC) en monocyte chemotactic protein-1 (MCP-1).

Tot op heden vervult serologie een centrale rol in de immunologische detectie van Q-koorts. Er wordt nog steeds onderzoek verricht ter validatie en optimalisatie van Q-koorts serologie [22-25]. In het licht van dalende serologische titers in de

jaren na infectie, en het aanhoudende debat over het afkappunt van serologische titers in de diagnostiek naar chronische Q-koorts, kan het meten van de cellulaire immuun respons – bijvoorbeeld met de IFN-γ test – waarschijnlijk van toegevoegde waarde zijn. Onderzoek hiernaar verdient verdere aandacht.

DEEL III. Immunosuppressiva en reumatoide artritis als risicofactoren voor chronische Q-koorts

Immunosuppressie is een risicofactor voor chronische Q-koorts, zoals blijkt uit dierstudies en beschrijvingen van infecties bij mensen. Echter, het type immunosuppressie dat het risico op chronische Q-koorts verhoogt, is niet goed gedefinieerd. Onze hypothese was dat het gebruik van TNF-blokkers (zoals remicade, adalimumab en humira) door patiënten met reumatoïde artritis (RA) en andere inflammatoire aandoeningen, een specifieke risicofactor is voor chronische Q-koorts. Dit is al aangetoond voor andere intracellulaire infecties, met name voor tuberculose. In **hoofdstuk 10** beschrijven we onze studie naar de prevalentie van anti-*C. burnetii* antistoffen bij RA patiënten met en zonder TNF-blokkers woonachtig in het Q-koorts endemisch gebied. In deze cohorten stelden we de diagnose chronische Q-koorts vast volgens de algemeen geldende Nederlandse richtlijn. We vonden een vergelijkbare prevalentie van *C. burnetii* infectie bij RA patiënten met en zonder TNF-blokkers. In de groep patiënten mét TNF-blokkers bleek 12,3% (7/57) van degenen met anti-*C. burnetii* antistoffen chronische Q-koorts te hebben, vergeleken met 5,5% (3/55) in de groep patiënten zonder TNF-blokkers (p=0,32). Vanwege het lage aantal chronische Q-koorts patiënten is de power van de studie beperkt en zou alleen een groot (>viervoudig) effect van TNF-blokkers op het risico op chronische Q-koorts aangetoond kunnen worden. Een interessante bevinding in deze studie was dat in een univariaat analyse van alle *C. burnetii*-geïnfecteerde RA patiënten (n=102) een associatie was te zien tussen gebruik van corticosteroïden en de ontwikkeling van chronische Q-koorts. Multivariaat analyse kon niet worden verricht vanwege het lage aantal chronische Q-koorts patiënten. Opvallend was, dat de prevalentie van chronische Q-koorts bij *C. burnetii*-geïnfecteerde RA patiënten veel hoger(8,9%) bleek te zijn dan eerder is gerapporteerd in een niet-geselecteerde geïnfecteerde populatie in hetzelfde gebied (1,6%) [23]. Dit impliceert dat RA en de behandeling ervan, met of zonder anti-TNF therapie, beschouwd moet worden als een risicofactor voor de ontwikkeling van chronische Q-koorts. Ons advies zou daarom zijn om in geval van *C. burnetii* infectie bij een patiënt met RA, zorgvuldige follow-up en analyse te verrichten met betrekking tot chronische Q-koorts.

In **hoofdstuk 11** presenteren we een casus van een RA patiënt met acute Q-koorts tijdens behandeling met TNF-blokkers, die chronische Q-koorts ontwikkelt nadat hij is geswitched naar behandeling met anti-B-cell (anti-CD20) monoklonale

antilichamen (rituximab). Deze casus leverde een aantal interessante inzichten op. We zagen dat bij deze patiënt anti-TNF therapie in de acute fase van Q-koorts de ontwikkeling van een serologische respons niet verhindert. Echter, een intacte humorale respons heeft de ontwikkeling naar chronische Q-koorts niet voorkomen, waarschijnlijk omdat de essentiële cellulaire immuun respons onderdrukt was tijdens de acute fase van de infectie. Alhoewel anti-B-cell therapie was ingezet na de acute Q-koorts episode, werd een toename van de anti-*C. burnetii* fase I antistoffen geobserveerd, wat leidde tot de diagnose chronische Q-koorts. Aangezien CD20-positieve B-cellen op dat moment uitgeschakeld waren door rituximab, nemen we aan dat reeds gevormde *C. burnetii*-specifieke memory B-cellen (die CD20-negatief zijn) verantwoordelijk zijn voor deze stijging van de antistoftiters. Door depletie van CD20-positieve B-cellen onderdrukt rituximab echter de respons op 'neo-antigenen'. Het is daarom te verwachten dat wanneer rituximab zou worden gebruikt op het moment van het eerste contact met *C. burnetii* (tijdens de acute infectie), er wel degelijk een serologische respons wordt verhinderd, en daarmee de diagnose van Q-koorts bemoeilijkt zou worden.

DEEL IV. Preventie van Q-koorts door vaccinatie tegen *C. burnetii*

Vaccinatie tegen *C. burnetii* kan mensen bescherming bieden tegen Q-koorts. Er is wereldwijd één humaan vaccin beschikbaar: Q-vax® (CSL, Victoria, Australia). Dit is een geïnactiveerd whole-cell vaccin. Het protocol van de fabrikant schrijft voor dat er pre-vaccinatie screening moet plaatsvinden. Deze screening bestaat uit een huidtest en serologie. Beide testen moeten worden verricht om vaccinatiekandidaten uit te sluiten met een (asymptomatische of niet-als-zodanig-herkende) doorgemaakte Q-koorts. Van deze mensen wordt aangenomen dat ze een hoger risico hebben op immunogemedieerde overgevoeligheidsreactie op de vaccinatie. Tijdens de Nederlandse Q-koorts uitbraak is door de autoriteiten besloten om vaccinatie aan te bieden aan mensen met cardiale en/of vasculaire risicofactoren voor chronische Q-koorts woonachtig in het Q-koorts endemisch gebied. Dit was een uniek besluit: *C. burnetii* vaccinatie was tot dan toe alleen toegepast bij mensen in Australië met beroepsmatig een verhoogd risico op *C. burnetii* infectie (o.a. slachthuis medewerkers, schaapscheerders, dierenartsen), hetgeen een veel jongere en gezondere groep mensen omvat.

In **hoofdstuk 12** beschrijven we de veiligheid en bijwerkingen van de Q-koorts huidtest en vaccinatie in de Nederlandse Q-koorts vaccinatie campagne. Vaccinatie van 1370 patiënten liet geen onverwachte bijwerkingen zien. Echter, 80% van alle gevaccineerden rapporteerden locale bijwerkingen, waarvan 26% heftig (volgens de algemeen geldende classificatie van vaccinatie bijwerkingen). Er werden 2 ernstige ongewenste voorvallen (serious adverse events 'SAE'; 0,1%) met een oorzakelijk verband vastgesteld. In beide gevallen ging het om een persisterende

subcutane zwelling op de injectie-plaats, zonder tekenen van een abces of granuloom. Hoewel we het niet met zekerheid kunnen concluderen, gaat het waarschijnlijk in beide gevallen niet om een immunologische respons tegen het vaccin, maar om een te diepe vaccinatie boven de insertie van de pees van de M. deltoïdeus.

Het voorkomen van locale bijwerkingen na de vaccinatie was geassocieerd met lokale bijwerkingen in de eerste dagen na de huidtest (met een negatieve aflezing van de huidtest na 7 dagen) en een hoge pre-vaccinatie *C. burnetii*-specifieke IFN-γ productie. De bijwerkingen vonden relatief meer plaats in vrouwen en bij jongere gevaccineerden. Bij gevaccineerden met lokale bijwerkingen na de huidtest en/of vaccinatie, was de post-vaccinatie immuun respons sterker (gemeten 6 maanden na vaccinatie). We concludeerden dat de Q-koorts vaccinatie veilig, maar reactief was in deze hoog-risico populatie met valvulaire / vasculaire risico factoren voor chronische Q-koorts.

In **hoofdstuk 13** onderzochten we in een steekproef van 260 gevaccineerden de post-vaccinatie immuun respons tegen *C. burnetii,* 6 en 12 maanden na vaccinatie. Na 6 maanden vonden we dat 46% van de gevaccineerden lage anti-*C. burnetii* antistoffen titers had en 67% een positieve IFN-γ test. Na 12 maanden was dit beide 60%. Ter vergelijking: serologische resultaten van 200 individuen met een natuurlijk doorgemaakte Q-koorts infectie waren positief in 99,5% van de gevallen, zowel 6 als 12 maanden na infectie, met hogere antistoffen titers. De immuun respons na Q-koorts vaccinatie is dus lager en beperkt tot een kleiner deel van individuen dan na een doorgemaakte Q-koorts infectie. Eerdere studies naar de immuun respons op hetzelfde Q-koorts vaccin bij Australische slachthuis medewerkers, schaapboeren en dierenartsen rapporteren een groter percentage seroconversie en cellulaire immuun respons na vaccinatie [26-28]. Dit suggereert dat er een verminderde immunogeniciteit is van het vaccin in onze oudere populatie met risico factoren voor chronische Q-koorts. Een interessante bevinding in deze studie was dat gevaccineerden met een positieve uitkomst in de IFN-γ test voorafgaand aan de vaccinatie een hoger percentage aan seroconversie lieten zien dan gevaccineerden met vooraf een negatieve uitkomst in de IFN-γ test: 74% versus 41% ($P<0.001$). Hieruit concludeerden we dat een positieve IFN-γ test voorafgaand aan vaccinatie in seronegatieve vaccinatiekandidaten waarschijnlijk duidt op pre-existente immuniteit, hetgeen resulteert in een versterkend effect van de vaccinatie.

Hoofdstuk 14 richt zich op de Q-koorts huidtest die, als een *in-vivo* intracutane test, een immuun respons kan uitlokken. We laten zien dat de huidtest, met een lage dosis van de Q-vax antigenen, bij ongeveer de helft van de individuen een

duidelijke *C. burnetii*-specifieke immuun respons veroorzaakt (seroconversie en/of stijging van de IFN-γ productie). Daarnaast hadden Q-koorts seropositieve individuen gemiddeld een verdubbeling van de antistoffen titer door de huidtest. Aangezien al deze individuen aanwijzingen hadden voor een pre-existente immuniteit tegen *C. burnetii* (ofwel een positieve huidtest, ofwel seropositiviteit), kan dit effect van de huidtest worden beschouwd als boosting. Een soortgelijk boosting effect is beschreven van de tuberculine huidtest op herhaaldelijke metingen van de interferon-γ release assay (IGRA) respons: deze testen worden vaak sequentieel gebruikt voor de diagnose van latente tuberculose [29]. Dit fenomeen maakt het lastig voor clinici om het onderscheid te maken tussen boosting en conversie bij een toename van de IGRA respons na een tuberculine huidtest. Op grond hiervan zouden clinici en epidemiologen op het gebied van Q-koorts zich ervan bewust moeten zijn dat individuen die een huidtest hebben gehad, zoals gebruikelijk is bij pre-vaccinatie screening, in de daaropvolgende metingen een hogere anti-*C. burnetii* antistoffen titer en een robustere IFN-γ productie zullen laten zien.

In **hoofdstuk 15** onderzochten we de dekkingsgraad van de Q-koorts vaccinatie campagne in Nederland in 2011. De campagne was bedoeld voor alle mensen met een risicofactor voor chronische Q-koorts woonachtig in het Q-koorts endemisch gebied. Prevalentie data van de gedefinieerde risicofactoren waren niet beschikbaar voorafgaand aan de campagne en er bestond geen geschikt registratiesysteem van deze aandoeningen in Nederland. Daarom was het vóór de start van de vaccinatie campagne lastig om een juiste inschatting te maken van de verwachte opkomst. Daarbij bleef het na afloop van de campagne onduidelijk hoeveel procent van de doelgroep was gevaccineerd. Het doel van onze retrospectieve cohort studie was om een hiervan een schatting te maken. Hiervoor identificeerden we allereerst het aantal mensen met de gedefinieerde risicofactoren in een steekproef van een Nederlandse huisartsen populatie-database, waaruit we de gestratificeerde prevalentie schattingen berekenden. Daarna extrapoleerden we deze prevalentie gegevens naar de populatie woonachtig in het Q-koorts epidemisch gebied. Dit leidde tot een schatting van ongeveer 12.000 mensen met een zekere of waarschijnlijke risicofactor voor chronische Q-koorts. Met behulp van de vaccinatie data van het aantal mensen uit dit gebied dat daadwerkelijk gescreened was voor Q-koorts vaccinatie, kwamen we tot een schatting van een totale dekkingsgraad van 11%. Gecorrigeerd voor het grotere aantal voor vaccinatie verwezen patiënten, was de geschatte dekkingsgraad 18%. Deze lage percentages moeten worden gezien in het licht van de complexiteit van deze doelgroep met vaak uitgebreide co-morbiditeit, en de vaccinatie procedure die een tweetal bezoeken aan een centrale GGD vereiste en gepaard ging met een invasieve pre-vaccinatie screeningsmethode. Bovendien

is bekend dat de dekkingsgraad van andere vaccinatie campagnes gericht op volwassen risico-groepen vaak teleurstellend laag is, zelfs na officiële en herhaaldelijke aanbevelingen. De hepatitis B vaccinatie voor mannen die sex hebben met mannen heeft bijvoorbeeld een geschatte jaarlijkse dekkingsgraad van 1% in Nederland [30]. Aangezien de Q-koorts epidemie in omvang afnam in de jaren na 2011, bleef de Q-koorts vaccinatie campagne beperkt tot 2011.

Algemene conclusies en toekomstig onderzoek

Meer kennis over immunologische aspecten van Q-koorts zal ons helpen bij het verbeteren van preventie, herkenning en behandeling van patiënten met Q-koorts. De studies in dit proefschrift hebben nieuwe inzichten opgeleverd over de immunologie van Q-koorts, maar er zijn nog veel vragen onbeantwoord gebleven en nieuwe vragen ontstaan.

Ons toekomstig onderzoek zal zich richten op het verder ontcijferen van de aangeboren afweer respons van de gastheer tegen *C. burnetii*. We zullen verder onderzoeken welke factoren in de afweer predisponeren voor inefficiënte klaring van de infectie en het ontstaan van chronische Q-koorts. Hoewel we hebben gevonden dat de IFN-γ respons niet defect is bij chronische Q-koorts patiënten, zouden patiënten die niet opknappen door langdurige antibiotische behandeling, gebaat kunnen zijn bij het toevoegen van immuno-stimulerende behandeling met IFN-γ. Met name bij chronische Q-koorts patiënten met persisterend lage *in-vtro* IFN-γ productie, zou het verhogen van de verdedigingsmechanismen van het afweersysteem door middel van IFN- γ therapie (Immukine) gunstig kunnen zijn. Een andere belangrijke vraag die nog antwoord behoeft, is hoe *C. burnetii* in staat is om te overleven in mononucleaire fagocyten op predelictie plaatsen zoals aangedane hartkleppen en de wand van aneurysmata. Een beter begrip van het lokale immunologische milieu in door *C. burnetii* geïnfecteerde aneurysmata/ hartkleppen, en de betrokken mechanismen die het weefsel beschadigen, vormen een mogelijke sleutel tot betere behandelopties voor chronische Q-koorts patiënten.

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Dankwoord

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Curriculum Vitae

Teske Schoffelen was born on March 29th 1985 in Tilburg. She attended high school at the Theresialyceum in Tilburg and received her diploma in 2003 (*cum laude*). She then started her medical studies at Radboud University Nijmegen. She followed a Masterclass in Internal Medicine at the Radboud university medical center (Radboudumc) in 2005, which raised her interests in this part of medicine. Next to her studies, she worked in elderly home care in Nijmegen. She followed courses at several other faculties of the university, among which the faculty of Arts where in 2006-2007 she obtained her first-year diploma History (*cum laude*). In 2010, she went to Indonesia for a 3-month clinical internship Low Income Countries supervised by dr. M. Keuter (NIIH, Nijmegen) and dr. H. Gasem (dr Kariadi Hospital, Semarang).

During her study, she developed a particular interest in research of infectious diseases and immunology. In 2007, She followed the course 'Molecular Biology and Toxicology', part of Biomedical Sciences. After that, she performed a short internship at the Department of Molecular Biology at the Nijmegen Centre for Molecular Life Science (NCMLS) under supervision of Prof. Stunnenberg, joining the research on Epigenetics of *Plasmodium falciparum* with Adriana Salcedo. In 2010, she performed an internship on 'Host recognition and cytokine response against *Cryptococcus gattii'* at the laboratory of Experimental Internal Medicine, Department of Internal Medicine, Radboudumc, under supervision of prof. dr. M.G. Netea and dr. T. Sprong.

In March 2011 she obtained her medical degree (*cum laude*). She then started her PhD research at the Department Internal Medicine, Radboudumc, under supervision of prof. dr. J.W.M. van der Meer, prof. dr. M.G. Netea, dr. ir. M. van Deuren and dr. T. Sprong. The subject of this research was immunology of Q fever, and the results are described in this thesis. During her PhD, she was a member of the clinical PhD council of the medical department of the Radboud University. For research collaboration, she visited the laboratory of prof. dr. Raoult and the laboratory of prof. dr. Mege at the Aix-Marseille University in Marseille. She worked at the Q fever laboratory of the Centers of Disease Control and Prevention (CDC) in Atlanta under supervision of dr. Gilbert Kersh.

In January 2015, she started her training in internal medicine (Radboudumc; supervisor Prof. J. De Graaf). She currently works as a resident internal medicine in the Slingeland hospital in Doetinchem. She will continue research on immunological aspects of Q fever in Radboudumc through research projects that are funded by Q-support.

List of publications

- **Schoffelen T**, Joosten LAB, Herremans T, de Haan AFJ, Ammerdorffer A, Rümke HC, Wijkmans CJ, Roest HI, Netea MG, van der Meer JWM, Sprong T, van Deuren M. Specific Interferon-γ detection for the diagnosis of previous Q fever. *Clin Infect Dis.* 2013 Jun;56(12):1742-51.
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- Keijmel SP, Raijmakers RR, **Schoffelen T**, Salet MCW, Bleeker-Rovers CP. A fatal case of disseminated chronic Q fever: a case report and review of the literature. *Submitted*

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